40473

National Library of Canada / Bibliothèque nationale du Canada

CANADIAN THESES ON MICROFICHE / THÈSES CANADIENNES SUR MICROFICHE

NAME OF AUTHOR/NOM DE L'AUTEUR PANG SOPHIE HELNG YU

TITLE OF THESIS/TITRE DE LA THÈSE Folate metabolism in Neurospora crassa
Glycine synthase and a mutant lacking cytosolic
serine hydroxymethyltransferase

UNIVERSITY/UNIVERSITÉ U of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Master of Science

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE February, 1979

NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. E.A. Cossins

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/DATÉ March, 1st, 1979 SIGNED/SIGNÉ Sophie Pang

PERMANENT ADDRESS/RÉSIDENCE FIXE 326, Southridge
10165 East 45 Avenue
Edmonton, Alberta

NL-91 (3-74)

National Library of Canada

Cataloguing Branch
Canadian Theses Division

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada

Direction du catalogage
Division des thèses canadiennes

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE

THE UNIVERSITY OF ALBERTA

FOLATE METABOLISM IN *NEUROSPORA CRASSA*. GLYCINE SYNTHESIS IN A MUTANT LACKING CYTOSOLIC SERINE HYDROXYMETHYLTRANSFERASE

by

© SOPHIE HEUNG YU PANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PLANT BIOCHEMISTRY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

SPRING, 1979

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "FOLATE METABOLISM IN *NEUROSPORA CRASSA*. GLYCINE SYNTHESIS IN A MUTANT LACKING CYTOSOLIC SERINE HYDROXYMETHYLTRANSFERASE." by SOPHIE HSUNG YU PANG in partial fulfillment of the requirements for the Degree of Master of Science in Plant Biochemistry.

.....
Supervisor

.....

.....

.....

DATED ..... February 13th 1979

ABSTRACT

Serine hydroxymethyltransferase activity was studied in *Neurospora crassa* wild type (FGSC No. 853) and a formate requiring mutant (FGSC No. 9). Two forms of the enzyme were fractionated and isolated from the cytoplasm and the mitochondria of the wild type. The cytosolic fraction of the mutant contained only trace amounts of enzyme activity but relatively large amounts were associated with the mitochondria. This situation was reversed in the wild type where greatest amounts of serine hydroxymethyltransferase were recovered in the cytosolic fraction.

The enzyme from both strains had an optimum pH of 8.0 in potassium phosphate buffer. In the wild type, the soluble enzyme was precipitated at 55-75% of saturation with $(NH_4)_2SO_4$ and following passage through Sephadex G-25, specific enzyme activity was increased by a factor of 55. The soluble enzyme of the wild type and the mitochondrial enzyme of the for mutant were subjected to chromatography on Sephadex G-200. The soluble enzyme of the wild type was collected in fractions 25 to 32 with peak activity in fraction 27. The mitochondrial enzyme of the mutant was, however, collected in fractions 30 to 35 with the peak activity in fraction 32. Interconvertibility of glycine and serine was demonstrated for both strains. However, the mitochondrial enzyme, unlike that of the cytosol, favoured serine formation. It, therefore, appeared that the mutant had little ability to form glycine from serine.

The origins of glycine in the formate mutant were, therefore,

investigated. Enzyme assays suggested operation of a pathway leading from isocitrate to glycine. For example, the levels of isocitrate lyase and L-alanine:glyoxylate transaminase were compared in the wild type and formate mutant. The specific activity of the lyase was approximately 250% higher in the formate mutant and that of the transaminase was 200% higher. Malate synthetase levels were also different but in this case the wild type contained about 12% more activity than the mutant.

Operation of the proposed route for glycine biosynthesis was examined in [1-^{14}C]glyoxylate feeding experiments. Time course studies showed that the labelled precursor was more rapidly incorporated into glycine by the mutant. In this strain, the specific radioactivity of glycine was up to 600% higher than that found in comparable experiments with the wild type. However, in the latter strain, more than twice as much glyoxylate carbon was associated with the organic acids.

Considering the established localization of these enzymes in *N. crassa*, it is suggested that glycine, formed in the glyoxysomes from isocitrate, is partially transported to the mitochondria for serine production. Methylenefolate needed in this synthesis could arise from utilization of exogenous formate.

ACKNOWLEDGEMENTS

I am very grateful to Dr. Edwin A. Cossins for his excellent guidance and suggestions during this study and in the preparation of this manuscript.

This thesis is specially devoted to my father and family whose constant encouragement and expectations never failed me.

The research contained in this thesis was supported by grants-in-aid of research from the National Research Council of Canada to Dr. E. A. Cossins.

TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
A. Comparison of *Neurospora crassa* and yeast folates	1
B. The generation of C-1 units	6
C. The major relationships between glycine and folate metabolism	7
D. The serine hydroxymethyltransferase reaction	9
E. The occurrence of serine hydroxymethyltransferase isoenzymes	11
F. Importance of the glyoxylate cycle in microbial metabolism	13
G. The present investigation	18
MATERIALS AND METHODS	19
Chemicals	19
Organism and methods of culture	19
Preparation of agar slants	20
Maintenance of stock cultures	20
Synthetic media	20
Conidia formation and harvesting	21
Spore germination in liquid culture	21
Sephadex column chromatography	22
Cell-free extracts	22
Partial purification of cell-free extracts	23
(a) Passage through Sephadex G-25 at 4°C	23
(b) Sephadex G-200 treatment	23
(c) Ammonium sulphate fractionation at 4°C	23
Density gradient centrifugation of cell-free extracts	25
Assay of serine hydroxymethyltransferase activity	27
(a) Methylene folate production from [3-^{14}C]serine	27
(b) Serine production from [2-^{14}C]glycine	27
Assay of isocitrate lyase activity	28
Assay of L-alanine:glyoxylate aminotransferase activity	29
Assay of malate synthetase activity	30

TABLE OF CONTENTS CONTINUED

	PAGE
Succinic dehydrogenase activity	30
Glyoxylate [1-^{14}C] feeding experiments	31
Free amino acid determinations	31
Estimation of protein contents	32
RESULTS	33
Effect of media supplements on growth of the formate mutant	33
Serine hydroxymethyltransferase activities of the wild type and *for* mutant	33
The presence of glyoxylate cycle enzymes in the wild type and formate mutant	49
(A) Isocitrate lyase activity	49
(B) L-alanine:glyoxylate transaminase activity	52
(C) Malate synthetase activity	52
Glyoxylate feeding experiments	52
DISCUSSION	58
General properties and localization of serine hydroxymethyltransferase	58
Possible functions of the mitochondria and soluble isoenzymes	60
Role of the glyoxylate cycle in glycine biosynthesis	64
Concluding remarks	66
BIBLIOGRAPHY	68

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1	Loss of Serine Hydroxymethyltransferase Activity after $(NH_4)_2SO_4$ Precipitation on Storage	39
2	Specific Enzyme Activities of Serine Hydroxymethyltransferase after Desalting with Sephadex G-25	40
3	Production of Glycine from Serine by Various Cellular Fractions of *N. crassa*	42
4	Interconversion of Glycine and Serine: *N. crassa* Wild Type and *for* Mutant	43
5	Malate Synthetase Activity: *N. crassa* Wild Type and *for* Mutant	55
6	Incorporation of [1-^{14}C]Glycolate by *Neurospora crassa*, Wild Type and *for* Mutant: Principal Products	56

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1	The Effect of pH on Serine Hydroxymethyltransferase Activity	35
2	The Relationship Between Serine Hydroxymethyltransferase Activity and Reaction Time	38
3	Density Gradient Centrifugation of Particulate Serine Hydroxymethyltransferase	46
4	Gel Filtration of Serine Hydroxymethyltransferase	48
5	Isocitrate Lyase Activity in *R. erasea* Wild Type and *for* Mutant	51
6	Glycine Production by L-Alanine:Glyoxylate Transaminase in *R. erasea* Wild Type and *for* Mutant	54

LIST OF SCHEMES

SCHEME	DESCRIPTION	PAGE
1	The primary structure of the folate cofactors	2
2	The major reactions for production of C-1 units	4
3	The glyoxylate cycle and glycine-serine biosynthesis ..	16
4	Isolation of crude mitochondrial pellets by differential centrifugation	24
5	Isolation of particulate fractions by density gradient centrifugation	26
6	A. Proposed one-carbon unit transport shuttle	62
	B. Possible glycine-serine relationships in *N. crassa*, wild type and *for* mutant	62

LIST OF ABBREVIATIONS

** 10-HCO-H_4PteGlu	= N^{10}-formyltetrahydropteroylmonoglutamate
5-CH_3-H_4PteGlu	= N^5-methyltetrahydropteroylmonoglutamate
5,10-CH_2-H_4PteGlu	= N^5,N^{10}-methylenetetrahydropteroylmonoglutamate
PteGlu	= Pteroylmonoglutamate
NAD	= nicotinamide adenine dinucleotide
EDTA	= disodium ethylenediaminetetra-acetic acid
Tris	= tris (hydroxymethyl) aminomethane
µCi	= microcurie
cpm	= counts per minute
DNTB	= 5,5 dithiobis

** The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the Biochemical Journal 102: 15 (1967).

The term 'folate' is used to designate pteroylglutamate derivatives in general.

INTRODUCTION

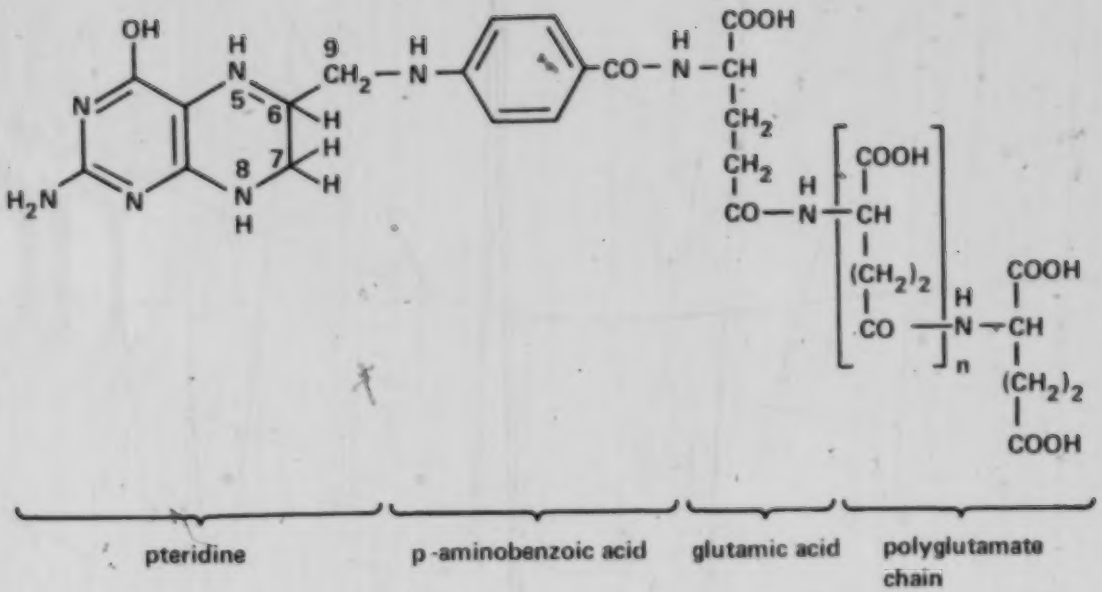
A variety of cellular constituents are formed by reactions involving addition of C_1 units. In the majority of such reactions, C_1 units are donated by highly reduced pteridine molecules related to folic acid.

Rabinowitz and Pricer (1957) demonstrated that folate derivatives are involved in the biosynthesis of carbons 2 and 8 of the purine ring. It is also clear that one-carbon units are needed in the synthesis of thymine, 5-hydroxymethylcytosine and 5-methyluridine (Whittaker and Blakley, 1961; Flaks and Cohen, 1959; Maley, 1962). Folates also play a vital role in polypeptide chain initiation via formylmethionine (Horikoshi and Doi, 1967; Dickerman *et al.*, 1967). Folates are also implicated in the *de novo* formation of methyl groups, with $5\text{-}CH_3\text{-}H_4PteGlu_n$ acting as an important methyl donor in the biosynthesis of methionine (Weissbach *et al.*, 1963; Kerwar *et al.*, 1966; Dodd and Cossins, 1970; Burton and Sakami, 1969).

As folate biochemistry has a number of nutritional and clinical implications much basic research has been carried out on bacterial and mammalian systems (Blakley, 1969). In addition, there have been a number of recent investigations of plant species (Cossins, 1978).

A. Comparison of *Neurospora crassa* and yeast folates.

Microbial, plant and animal cells contain principally polyglutamyl folates which are either formylated, methylated or unsubstituted (see Scheme 1 and 2).

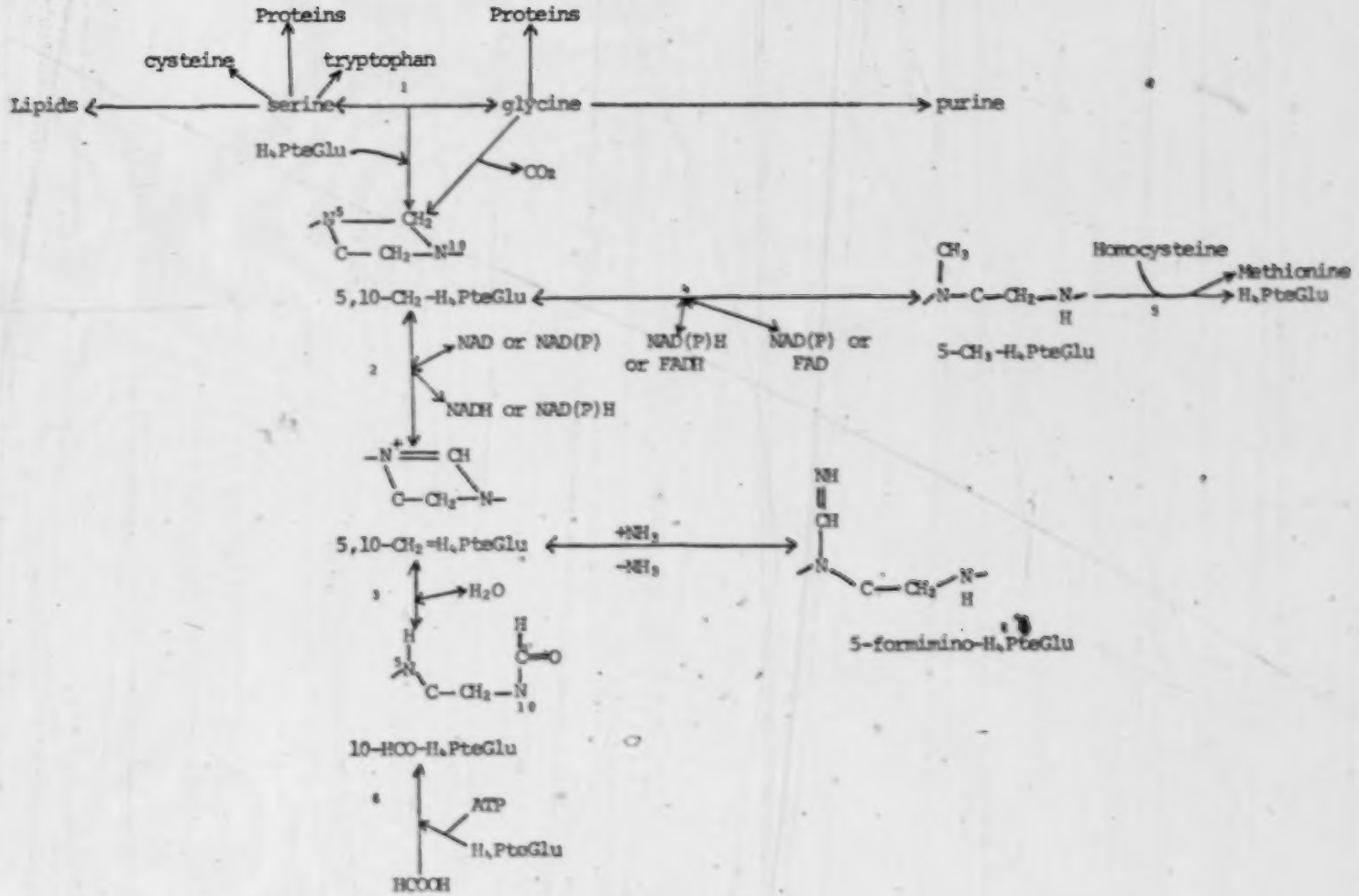

pteridine
p-aminobenzoic acid
glutamic acid
polyglutamate chain

SCHEME 1

SCHEME 2

The Major Reactions for Production of C-1 Units

Enzyme	EC Number	Reaction
Serine hydroxymethyltransferase	2.1.2.1	1
5,10-methylenetetrahydrofolate dehydrogenase	1.5.1.5	2
5,10-methenyltetrahydrofolate cyclohydrolase	3.5.4.9	3
5,10-methylenetetrahydrofolate reductase	1.1.1.68	4
5-methyltetrahydrofolate homocysteine transmethylase	2.1.1	5
10-formyltetrahydrofolate synthetase	6.3.4.3	6


Proteins
Proteins
cysteine
tryptophan
Lipids
serine
glycine
purine
H4PteGlu
CO2
5,10-CH2-H4PteGlu
NAD or NAD(P)
NADH or NAD(P)H
NAD(P)H or FADH
NAD(P) or FAD
5-CH3-H4PteGlu
Homocysteine
Methionine
H4PteGlu
5,10-CH=H4PteGlu
+NH3
-NH3
H2O
5-formimino-H4PteGlu
10-HCO-H4PteGlu
ATP
H4PteGlu
HCOOH

The folates of *Saccharomyces cerevisiae* were investigated by Schertel *et al.* (1965) who revealed a distribution of 63% 10-$HCOH_4PteGlu_n$, 14% 5-$HCOH_4PteGlu_n$ and 20% 5-$CH_3H_4PteGlu_n$. Other studies elucidated the polyglutamyl chain lengths of yeast folates. Thus Basset *et al.* (1976) reported mostly heptaglutamates (67-71%), hexaglutamates (12-16%) and octoglutamates (10-13%) for a brewer's strain of *S. cerevisiae*. With baker's yeast, Baugh *et al.* (1975) reported a distribution of 58% hexaglutamate and 10% heptaglutamate derivatives. In other studies, Lor and Cossins (1972) examined actively growing cultures of *S. cerevisiae* (ATCC 9763). They found that L-methionine supplements to a defined growth medium reduced the concentrations of various methylfolates. Smaller decreases also occurred in the pools of formyl and unsubstituted folates.

The folates of *Neurospora crassa* are also highly conjugated, occurring as formyl and methyl derivatives of $H_4PteGlu$ (Cossins *et al.*, 1975, 1976). Studies of serine- and formate-requiring mutants showed that native folate pools were drastically altered by genetic lesions which prevent synthesis of key folate-dependent enzymes. In this regard, Cossins *et al.* (1976) showed that a formate mutant, partially deficient in serine hydroxymethyltransferase, contained less methyl but more formyl folates than the wild type. An interesting example of methionine auxotrophy in *N. crassa*, (met-6), is clearly related to a lesion in polyglutamyl folate synthesis (Selhub *et al.*, 1969). By analogy with other biochemical pathways, it follows that analysis of these and related mutants could provide additional knowledge of folate metabolism. Such studies could hopefully lead to a better understanding of the interrelationships between key enzymes of folate metabolism

(Scheme 2) and aid in the elucidation of the major mechanisms for control of C_1 unit generation.

B. The generation of C-1 units.

The one-carbon unit of native folates is subjected to oxidation and reduction as summarized in Scheme 2. Enzymes catalyzing such reactions, generally use pyridine nucleotides as donors or acceptors of electrons and much is known of their catalytic properties (Blakley, 1969).

Perhaps the major reaction for production of one carbon units is that catalyzed by serine hydroxymethyltransferase (Equation 1).

$$\text{Serine} + H_4\text{PteGlu} \rightleftarrows \text{glycine} + 5,10\text{-}CH_2\text{-}H_4\text{PteGlu} \qquad (1)$$

The enzyme is probably ubiquitous to living cells (Blakley, 1969) and in some instances has importance in serine biosynthesis (Tolbert, 1971).

Glycine is also an important source of one-carbon units in many species (Yoshida and Kikuchi, 1970). For example, in bacterial mutants lacking serine hydroxymethyltransferase (Newman and Miller, 1974) this amino acid can apparently provide all of the C_1 units needed for growth. In such species glycine decarboxylase provides 5,10-methylene-H_4PteGlu in a complex reaction (Equation 2).

$$\text{Glycine} + \text{NAD} + H_4\text{PteGlu}_n \rightleftarrows 5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n + \text{NADH} + CO_2 + NH_3 \qquad (2)$$

This important reaction will be discussed in more detail under section C of this Introduction.

Considering the central importance of 5,10-CH_2-H_4PteGlu$_n$, it is not surprising that work on its metabolism has been extensive (*e.g.*

Blakley, 1969; Shane and Stokstad, 1977). This compound is known to be utilized in the biosynthesis of thymidylate, and is also converted to 5-methyl-H_4PteGlu by 5,10-methylenetetrahydrofolate reductase, a reaction of importance in methionine biosynthesis. Alternatively, a dehydrogenase can oxidize this folate to 5,10-methenyltetrahydropteroyl-polyglutamate, which is in turn a precursor of 10-formyltetrahydro-pteroylpolyglutamate. In several organisms, the latter folate is an important formyl donor and C_1 units from this source commonly enter the purine ring and f-met-tRNA (Whittaker and Blakley, 1961; Flaks and Cohen, 1959; Maley, 1962; Horikoski and Doi, 1967; Dickerman *et al.*, 1967).

Several species generate C_1 units in the formyltetrahydro-folate synthetase reaction (Blakley, 1969; Harvey and Dev, 1974). This reaction has clear physiological importance (Rabinowitz and Pricer, 1958, 1962; Nowak and Himes, 1971; Curthoys and Rabinowitz, 1971) and therefore represents a complementary source of C_1 units. Evidence is accumulating that some photosynthetic organisms may generate formyl-folate via this reaction (Lor and Cossins, 1978) but more research is needed to elucidate such contributions in higher plants.

Other less important sources of C_1 units include formaldehyde (Seigel and Lafaye, 1950), the formimino group of formiminoglutamic acid (Taiver and Wyngarden, 1959) and carbon dioxide (Jungermann *et al.*, 1968; Ljungdahl and Wood, 1969; Parker *et al.*, 1971).

C. The major relationships between glycine and folate metabolism.

There is excellent evidence that the α-carbon of glycine is a common precursor of the β-carbon of serine in bacteria (Osborne *et al.*,

1960; Nash, 1953; Van Dyke *et al.*, 1970), plants (Bruin *et al.*, 1970; Clandinin and Cossins, 1972) and animals (Yoshida *et al.*, 1969; Yoshida and Kikuchi, 1970).

In this connection, the work of Yoshida and Kikuchi (1970) showed that a glycine cleavage enzyme was widely distributed in nature. In various species of mammals and birds this enzyme is exclusively mitochondrial. This reaction is freely reversible but most physiological conditions appear to favour glycine catabolism (Equation 2). In this respect, C_1 units derived from glycine, are further oxidized to CO_2 in the inner mitochondrial membrane (Yoshida *et al.*, 1969; Tada *et al.*, 1969; Yoshida and Kikuchi, 1970). In other organisms, coupling of the cleavage reaction to serine hydroxymethyltransferase (Equation 1) results in one molecule each of serine, CO_2 and NH_3 being formed from two molecules of glycine (Tolbert, 1962; Tolbert *et al.*, 1968; Kisaki *et al.*, 1969; Bruin *et al.*, 1970).

The importance of glycine decarboxylase in yeast folate metabolism is inferred by the work of Ogur *et al.* (1977). In this study, a new mutation introducing a one-carbon requirement (*e.g.* formate) for growth on glycine supplemented medium of a serine-glycine auxotroph (ser-1) was correlated with a lack of glycine decarboxylase activity.

In *N. crassa*, there is good evidence for glycine cleavage in the wild type and C_1 units derived from this source are incorporated into a variety of products (Cossins *et al.*, 1976). The formate mutant (FGSC 9) lacks this glycine cleavage reaction and consequently fails to grow on minimal medium supplemented with this amino acid.

D. The serine hydroxymethyltransferase reaction.

Serine hydroxymethyltransferase (EC 2.1.2.1) which catalyzes the reversible formation of 5,10-CH_2-H_4PteGlu and glycine from serine (Equation 1) is considered the major source of C_1 units in the folate pool of most procaryotes and eucaryotes. Evidence has accumulated that this enzyme is responsible for both the biosynthesis and catabolism of serine. It, therefore, follows that some organisms synthesize glycine by this route. For example, Wilkinson and Davies (1958) demonstrated glycine production from serine by dialyzed turnip extracts. The reaction was reversible and H_4PteGlu-dependent. In contrast, Hauschild (1959) found that the enzyme of *Zea mays* seedlings favoured serine formation. More recently, interest has centered on the role of this reaction in serine synthesis during operation of the glycollate pathway (Tolbert, 1971).

Serine hydroxymethyltransferase has been studied from a number of microbial sources including *Salmonella typhimurium* (Stauffer *et al.*, 1974), *Escherichia coli* (Miller and Newman, 1973), *S. cerevisiae* (Botsford and Parks, 1969), and *N. crassa* (Burton and Metzenberg, 1975). Several of these recent studies have employed mutant strains which have allowed some elucidation of the interrelationships between this reaction and other pathways of C_1 metabolism.

The regulation of serine hydroxymethyltransferase is of recent interest. Results of such work suggest that complex and variable mechanisms exist in different species. For example, Stauffer *et al.* (1974) examined control of this enzyme in *Salmonella typhimurium*. Their results indicated that limitation of a methionine auxotroph for methionine did not cause derepression of this enzyme as occurs in

Escheriohia coli (Greene and Radovich, 1975). However, a 6-fold decrease in specific activity was observed when *S. typhimurium* cells were grown in glucose minimal medium supplemented with serine, glycine, methionine, adenine, guanine and thymine. None of these compounds added to the growth medium individually produced more than 42% reduction of wild type enzyme activity. This enhanced repression by a combination of compounds suggested a form of cumulative repression of enzyme synthesis. Growth of serine and thymine auxotrophs, with the respective requirement of each limiting, did not result in increased enzyme activity. However, growth of a purine auxotroph with a limiting amount of either guanine or inosine resulted in a 5- to 7-fold increase in enzyme activity. In *E. coli* K12, serine hydroxymethyltransferase levels are decreased by growth in glycine (about 40 mM) and by a mixture of C_1-derived metabolites such as histidine, methionine, adenine, guanine, and thymidine at moderate concentrations (Miller and Newman, 1973).

Enzyme studies of methionine auxotrophs of *S. cerevisiae* revealed that serine hydroxymethyltransferase is the first enzyme in the synthesis of the methyl group of methionine (Botsford and Parks, 1969). Serine hydroxymethyltransferase and the methylation of homocysteine were not repressed by methionine and were stimulated by glycine. The authors concluded that glycine rather than methionine was involved in the control of enzyme synthesis.

In *N. crassa*, regulation of serine hydroxymethyltransferase, methylenetetrahydrofolate reductase and methyltetrahydropteroylpolyglutamate:homocysteine methyltransferase have been investigated (Burton and Metzenberg, 1975). Addition of choline to the culture medium decreased the specific activity of these enzymes (Burton and Metzenberg,

1975). Methionine appeared to potentiate the choline effect, but when added alone was without effect. Neither choline, methionine, nor S-adenosylmethionine appeared to be the immediate corepressor of synthesis of these enzymes. In other studies of *N. crassa*, Cossins *et al.* (1976) compared the concentrations of serine hydroxymethyltransferase in the wild type and a formate mutant (FGSC 9) during exponential growth on defined minimal media. The mutant contained very low levels of serine hydroxymethyltransferase but in the wild type, exogenous (1 mM) glycine increased the specific enzyme activity. Such supplements also increased folate concentrations by up to threefold. To examine these effects in more detail, these workers supplied various potential C_1 unit donors during exponential growth. It was concluded that glycine enhanced C_1 unit turnover in the wild type. The partial deficiency of the formate mutant in serine hydroxymethyltransferase and inability to cleave glycine suggested that an alternative pathway must exist for generation of this amino acid.

E. The occurrence of serine hydroxymethyltransferase isoenzymes.

Mazelis and Liu (1967) obtained some evidence for two forms of serine hydroxymethyltransferase during purification of the cauliflower enzyme. Two pH optima were observed when production of ^{14}C-labelled methylene folate was monitored with [3-^{14}C]serine as substrate. Preliminary gel filtration also resulted in a partial separation of two enzyme active peaks.

Nakano *et al.* (1968) and Fujioka (1969) have carried out extensive work on mammalian serine hydroxymethyltransferases. It is clear that distinct mitochondrial and cytosolic enzymes exist in rat

liver (Nakano *et al.*, 1968). For rabbit liver, Fujioka (1969) found that both isoenzymes favour serine catabolism although the reverse reaction was detected in appreciable amounts. Chasin *et al.* (1974) found that in Chinese hamster cells, a glycine auxotrophic mutant lacked the mitochondrial isoenzyme. It was also clear that loss of glycine auxotrophy by reversion was accompanied by development of the mitochondrial enzyme. This strongly suggested that the particulate isoenzyme was mainly responsible for glycine biosynthesis.

In spinach and tobacco, isoenzymes of serine hydroxymethyltransferase are associated with the cytosolic and mitochondrial fractions (Kisaki *et al.*, 1971). However, in spinach leaves, the enzyme is mainly associated with the cytoplasm.

Work on *S. cerevisiae* has also given evidence for serine hydroxymethyltransferase isoenzymes (Zelikson and Luzzati, 1976). As in liver, one isoenzyme is mitochondrial and the other is cytoplasmic. The former is, however, lacking in a thymidylate-less yeast mutant which again implies a compartmentation of function within the eucaryotic cell.

Physical and catalytic differences also exist between the isoenzymes of *S. cerevisiae*, especially with respect to chromatographic behaviour, stability, pH optima, and apparent Michaelis constants for tetrahydrofolic acid (Zelikson and Luzzati, 1976). Similar differences in stability, chromatography and electrophoretic mobility were found (Nakano *et al.*, 1968; Palekar *et al.*, 1973) for the isoenzymes purified from rat liver. Mechanistic and stereochemical studies showed that the cytoplasmic enzyme was also capable of catalyzing an aldol cleavage of allo-threonine and decarboxylation of aminomalonate. In contrast, the mitochondrial enzyme catalyzed only the serine-glycine interconversion

(Palekar *et al.*, 1973). However, both isoenzymes, purified from rabbit liver, exhibited similar ratios of serine hydroxymethyltransferase/threonine (allothreonine) aldolase activities (Akhtar *et al.*, 1975). The rabbit liver isoenzymes showed remarkable differences in their sensitivity to inhibitors such as chloroacetaldehyde or glycinaldehyde (Akhtar *et al.*, 1975). These last properties do not appear to have been studied in yeast.

The loss of the mitochondrial isoenzyme leads to a general requirement for dTMP, methionine, adenine and histidine in the thymidylate-less yeast mutant. This may imply that the mitochondrial isoenzyme is physiologically active in providing methylenetetrahydrofolate for these syntheses. Moreover, there are indications that folate interconverting enzymes and the formyltetrahydrofolate:Met-tRNA transformylase are mitochondrial in yeast (Halbreich *et al.*, 1971). More work is clearly needed on the control and the metabolic roles of these serine hydroxymethyltransferase isoenzymes in fungi.

F. Importance of the glyoxylate cycle in microbial metabolism.

The ability of many microorganisms to grow on 2-carbon compounds such as acetate or ethanol as the only carbon source is now clearly linked to the anaplerotic function of the glyoxylate cycle (Kornberg and Krebs, 1957). The net result of cycle operation is that two molecules of acetyl-coenzyme A are combined into one molecule of succinate which then functions as a precursor in gluconeogenesis.

Duntze *et al.* (1969) studied the regulation and localization of the cycle enzymes in *S. cerevisiae*. These authors found that all specific enzyme activities increased when growth conditions favoured an anaplerotic pathway. In yeast, isocitrate lyase and malate

synthase are located in the cytoplasm but succinate dehydrogenase is found only in the mitochondrial fraction (Duntze *et al.*, 1969).

In other work, glyoxylate cycle enzymes were found in *Aspergillus tamarii* growing on ethanol (Graves *et al.*, 1976). In this case malate synthase and isocitrate lyase were localized in the microbodies. These organelles also occurred in greater densities when hyphae were grown on media supplemented with polypectate, carboxymethyl cellulose (*e.g. Pythium ultimum, Sclerotium rolfsii, Whetzelinia sclerotiorum*) or ethanol (*e.g. Rhizopus arrhizus*). Thus, a correlation was established between microbody frequency and assimilation of these substrates. The microbodies of *A. tamarii*, growing on ethanol, are probably glyoxylate cycle sites as in higher plants (Beevers, 1969), various protozoa (Miller *et al.*, 1968; Graves *et al.*, 1972), yeast (Szabo and Avers, 1969) and *Blastocadiella emersonii* (Mills and Cantino, 1975).

Support for a glyoxysome-like function for the microbodies of these filamentous fungi growing on 2-carbon substrates is provided by the finding that various species show elevated levels of glyoxylate-cycle enzymes (Flavell and Fincham, 1968; Maxwell *et al.*, 1975) under these conditions. Kobr *et al.* (1969) succeeded in isolating glyoxysome-like particles from acetate-grown *N. crassa* and these organelles contained appreciable levels of isocitrate lyase and malate synthase.

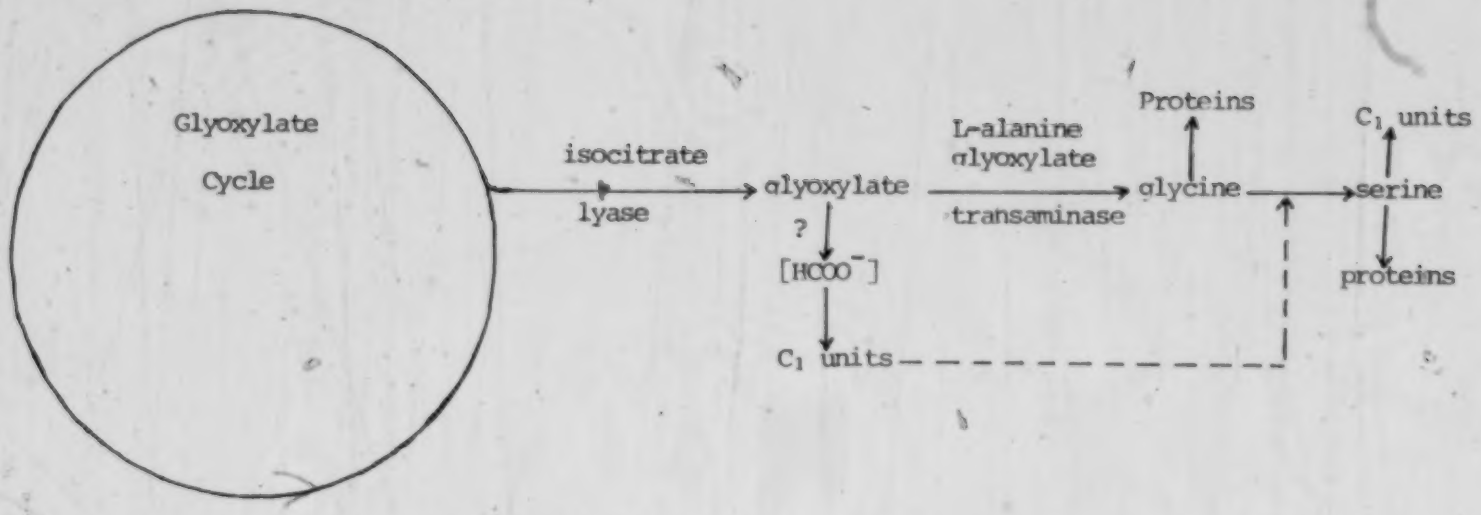
The photosynthetic bacterium *Rhodopseudomonas capsulata* has enzymes of the phosphorylated and non-phosphorylated pathways for serine-glycine synthesis (Beremand and Sojka, 1977). These workers found that *Ser*-1 and *Ser*-2 mutants grew well on malate medium supplemented with glyoxylate. This suggested that *R. capsulata* possesses

ability to form serine and glycine by the glyoxylate route as depicted in Scheme 3.

The formation of glycine by transamination of glyoxylic acid has been reported in a *N. crassa* mutant requiring glycine or serine by Wright (1951). This author demonstrated that glyoxylate could replace glycine in the growth of this *Neurospora* mutant.

Cell-free extracts of a *Pseudomonad* strain 2RCC-1 also synthesized glycine from alanine, aspartic acid, glutamic acid, asparagine and glutamine by transamination with glyoxylic acid (Campbell, 1955). The transamination reaction was pyridoxal phosphate-dependent, requiring 20 µg per ml for full activity. The optimum pH range for activity was from 8.0 to 9.0. Combepine (1969) also detected such a transamination reaction in *N. crassa* wild type cultured with various carbon sources.

McCurdy and Cantino (1960) followed the synthesis of isocitrate lyase and glycine-alanine transaminase during the development of both colorless and resistant-sporangial plants in the aquatic fungus, *Blastocladiella emersonii*. They found that isocitrate lyase and glyoxylate-alanine transaminase were key components of both the light-stimulated growth of OC plants (ordinary colorless) and in the bicarbonate triggered morphogenesis of RS plants (resistant sporangial). Cantino and Horenstein (1959) found that glyoxylate and succinate together, direct products of the isocitratase cleavage, will replace the light effect *in vivo*. This also suggested that there is a direct metabolic route from glyoxylate to glycine. Kikuchi *et al.* (1958) reported an enzymatic reaction in *Blastocladiella* yielding δ-aminolevulinic acid



SCHEME 3. The glyoxylate cycle and glycine-serine biosynthesis.

Dotted line depicts possible source of methylene folate in mutants partially deficient in serine hydroxymethyltransferase.

from succinate and glycine which was stimulated by light. Moreover, studies with radioactive tracers have implicated δ-aminolevulinic acid as an intermediate in the conversion of glycine to thymine and purines (Shemin, 1956), as well as carotene synthesis (Mackinney *et al.*, 1955). Cantino (1960) observed that thymine will substitute for light in DNA synthesis in *Blastocladiella*. McCurdy and Cantino (1960) suggested that δ-aminolevulinic acid is an important link connecting glyoxylate, glycine and succinate with light stimulated growth and, in RS morphogenesis, with carotene synthesis.

It was mentioned earlier that the microbodies of fungi assimilating 2-carbon substrates contain enzymes of the glyoxylate cycle. It is also likely that the microbodies of such fungi utilizing other substrates would contain a different set of enzymes. For example, the microbodies of *Candida boidinii* (Roggenkamp *et al.*, 1975) growing on methanol contain an alcohol oxidase and catalase which are crucial in allowing the yeast to use methanol as a sole carbon and energy source. Numerous other fungi are also reported to contain abundant microbodies under conditions not normally inducive for the glyoxylate cycle, *e.g.* *Whetzelinia sclerotiorum* growing on carboxymethyl cellulose and *Sclerotium rolfsii* growing on polypectate (Maxwell *et al.*, 1975), or the inter- and intracellular stages of such parasitic fungi as *Cymadothea trifolii* (Camp and Whittingham, 1972), *Fusarium oxysporum* (Wergin, 1972), and *Cladosporium cucumerinum* (Laborda and Maxwell, 1976). Elucidation of the pathways found in the microbodies of these fungi could be important to an understanding of the mechanisms which enable them to exploit diverse habitats.

In a study of *S. cerevisiae*, Duntze *et al.* (1969) provided

evidence that the glyoxylate cycle, although operating in the cytoplasm, has close functional relationships with mitochondrial metabolism. Thus succinate, provided by the cycle, could not be used as a precursor of oxaloacetate in the cytoplasm but had to enter the mitochondria where reduction via succinate dehydrogenase occurred.

G. The present investigation.

As noted above, the serine hydroxymethyltransferase reaction in *N. crassa* appears to function in the direction of C_1 unit generation and as such represents a major route for methylene folate synthesis. The existence of formate-requiring mutants (Harrold and Fling, 1952) and the subsequent demonstration of their serine hydroxymethyltransferase deficiency (Cossins *et al.*, 1976; Burton and Metzenberg, 1975) suggest that C_1 units can readily arise through the 10-formyltetrahydrofolate synthetase reaction. Under such conditions it follows that glycine precursors, other than serine, must also be utilized.

The present study has, therefore, examined the formate mutant (C-24) isolated by Harrold and Fling (1952) in more detail with particular emphasis on the possible origins of glycine. The finding that this mutation resulted in loss of a cytosolic serine hydroxymethyltransferase (Pang and Cossins, 1978) raises a number of basic questions regarding compartmentation of folate metabolism in *N. crassa*. Furthermore, the mutant appeared to generate glycine from glyoxylate via the partial reactions of the glyoxylate cycle. Surprisingly, glyoxylic acid could not replace the formate requirement for growth. Isocitrate lyase does not, therefore, generate a direct precursor of C_1 units in this mutant.

MATERIALS AND METHODS

Chemicals

Tetrahydrofolic acid was purchased from Sigma Chemical Co., St. Louis, U.S.A. L-[3-^{14}C]serine and [2-^{14}C]glycine were purchased from Amersham/Searle, Chicago, U.S.A. [1-^{14}C]Glyoxylate was purchased from Amersham, England. dl-L-Tetrahydrofolic acid (1.6 μmoles) in β-mercaptoethanol (40 μmoles) was prepared by taking 35 μl of mercaptoethanol (M. Wt. 78.13) in 10 ml of H_2O. A 2.7 ml aliquot of this solution together with 0.2 ml of 1N NH_4OH were added to a vial containing 25 mg of dl-L-tetrahydrofolic acid. One hundred μl of the above solution gave 1.6 μmoles of THFA.

Sephadex gels and blue dextran 2,000 were obtained from Pharmacia Canada Limited, Montreal, Canada. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-bis-[4-methyl-5-phenyloxazol-2-yl]-benzene (dimethylPOPOP) were purchased from Nuclear-Chicago, Des Plaines, U.S.A. Bacto-agar was routinely supplied by Difco Laboratories, Detroit, U.S.A. All other chemicals were obtained from Fisher Scientific Company, Edmonton. In every instance, the chemicals used were of the highest purity available.

Organism and Methods of Culture

Neurospora crassa, Lindegren A wild type (FGSC no. 853), was maintained in slope culture on defined minimal medium as described below. A formate-requiring mutant, strain C-24 (FGSC no. 9) was maintained in the same medium supplemented with 10 mM sodium formate.

Preparation of Agar Slants.

Ten gm of bacto-agar were suspended in 500 ml of demineralized water and steamed for 10 minutes with constant stirring. Ten ml of the resulting solution were then pipetted into 20x150 mm test-tubes, plugged with absorbent cotton and autoclaved for 10 min at 15 psi. The tubes were tilted to form slants and allowed to cool. After an incubation period of 24 hr at 30°C, any slants which showed growth were discarded. The remainder were stored in the refrigerator at 4°C for subsequent inoculation.

Maintenance of Stock Cultures.

Stock was routinely maintained by transferring conidia to new agar slants every 4 weeks. Such transfers were incubated at 30°C for 2-3 days and followed by two days in the light (Davis and DeSerres, 1970). Then they were stored in the refrigerator at 4°C.

Synthetic Media.

Vogel's medium N is now the most widely used minimal medium for vegetative cultures of *N. crassa*. The salt mixture can be stored indefinitely as a 50-strength solution, and was prepared as follows:

The following ingredients were added to 750 ml of distilled water, Na_3citrate$\cdot 5H_2O$, 150 g; KH_2PO_4, 250 g; NH_4NO_3, 100 g; $MgSO_4 \cdot 7H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$ (predissolved in 20 ml H_2O, added slowly), 5 g; biotin solution, 5 ml; and trace element solution (see below), 5 ml, allowing each reagent to dissolve before addition of the next.

The final volume of the solution was then adjusted to 1 litre and chloroform (2-3 ml) was added as a preservative. The solution was stoppered and stored at room temperature.

Single-strength medium (medium N) was made by adding 1 part

stock solution to 49 parts distilled water, adding 1.5% sucrose as a carbon source, and autoclaving. The final pH was 5.8.

A trace element stock solution was prepared by adding the following ingredients successively, with stirring, to 95 ml of distilled water: citric acid.$1H_2O$, 5 g; $ZnSO_4 \cdot 7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 25 mg; $MnSO_4 \cdot 1H_2O$, 5 mg; H_3BO_3, 5 mg; $Na_2MoO_4 \cdot 2H_2O$, 5 mg. The final volume was adjusted to 100 ml. After addition of chloroform (1 ml) as a preservative, the solution was stored at room temperature.

A biotin solution was prepared by dissolving 5 mg of biotin in 100 ml of 50% (v/v) ethanol and was stored in the refrigerator.

Conidia Formation and Harvesting.

For production of conidia, 500 ml batches of the defined minimal medium (Vogel, 1964) were supplemented with 2% (w/v) sucrose as carbon source and 2% (w/v) agar. This medium, contained in 1 litre Fernbach flasks, was inoculated with fresh conidia and incubated in darkness at 30°C for two days. This was followed by a 3-day period of aeration in light (Davis and DeSerres, 1970) to induce conidiospore production. Conidiospores were harvested under aseptic conditions by washing the cultures with sterile water, followed by filtration through four layers of fine cheesecloth. The orange solution containing the conidia was filtered then centrifuged at 2,500 g for 10 min. After making up to 50 ml, aliquots of these were used for inoculation of liquid media.

Spore Germination in Liquid Culture.

For liquid culture of the formate mutant, the defined medium was supplemented with 10 mM formate. Such cultures (1½ litres)

routinely contained 25 g of sucrose and 25 ml of 50-strength stock Vogel's medium. After inoculation, the culture was vigorously aerated with sterile air and maintained at 25°C for periods up to 27 hr (Cossins *et al.*, 1976). Under these conditions mycelial growth was exponential 18 hr after inoculation. Mycelial samples were harvested by rapid filtration. Dry weights were determined after washing with acetone as described by Davis and DeSerres, 1970).

Sephadex Column Chromatography.

Sephadex G-25 from the supplier was suspended in distilled water and boiled for 1 hr with constant stirring. Fine particles were removed by decantation and the gel was degassed *in vacuo* to remove air bubbles. Columns were filled with gel under gravity to give bed volumes of 1.6x20 cm. The columns were then washed with 200 ml of 0.01 M potassium phosphate buffer (pH 7.5) and kept at 4°C until required.

Sephadex G-200 was soaked in 0.01 M potassium phosphate buffer (pH 7.5) for 3 days at 4°C with constant stirring. After removal of fines and air bubbles the gel was packed into a column giving a final bed volume of 2.8x51 cm. The columns were then kept at 4°C until required.

Cell-free Extracts.

At the end of the desired culture period, mycelia were collected by rapid filtration and washed with 100 ml of 0.44 M sucrose in AMT buffer (0.1 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M tris) at 4°C (Kuntzel and Noll, 1967). All subsequent operations were carried out at 4°C. The hyphal mass was then mixed with 10 gm of acid-washed sea sand and ground thoroughly in 30 ml of sucrose-AMT. The homogenate was centrifuged for 10 min at 500 g and sedimented material was washed once with 25 ml sucrose-AMT. The combined supernatants were then centrifuged for

20 min at 26,000 g to yield a mitochondrial pellet. The post-mitochondrial supernatant was designated cytoplasmic fraction and the crude mitochondrial pellet was resuspended in sucrose-AMT.

Partial Purification of Cell-free Extracts.

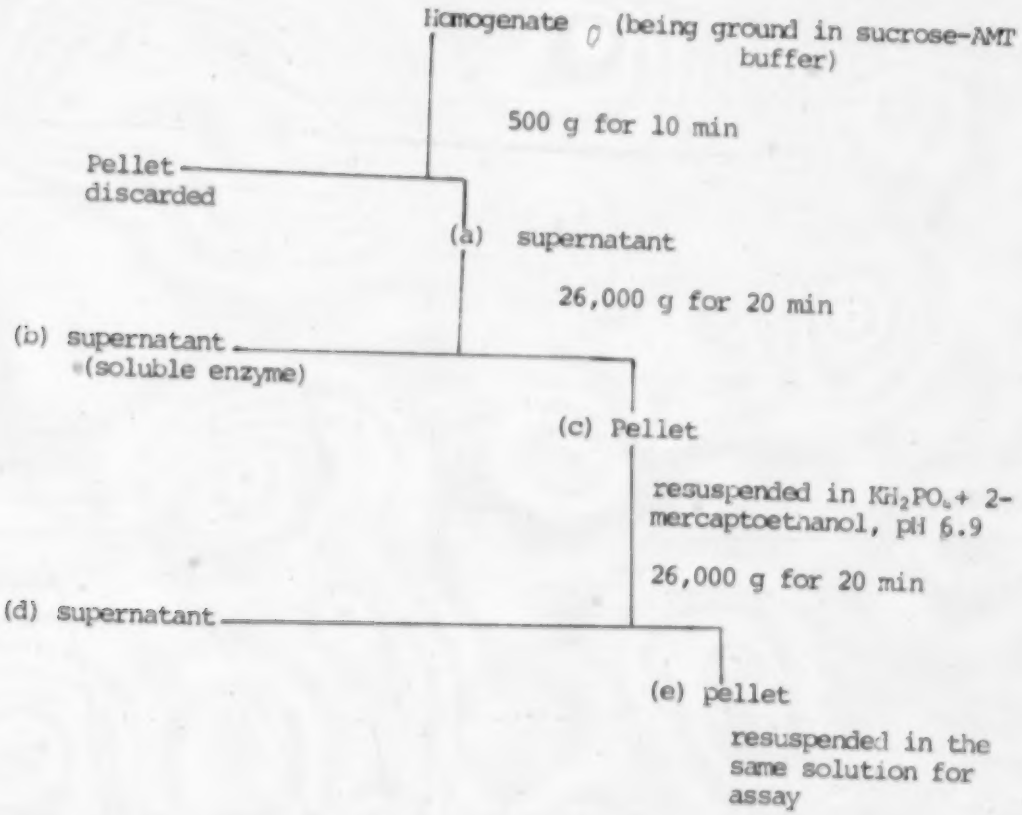
a) Passage through Sephadex G-25 at 4°C. The cell-free extracts (8 ml) was applied to a 1.6x20 cm column of Sephadex G-25 and eluted with 0.01 M potassium phosphate (pH 7.5). Fractions of 2 ml were collected at a flow rate of 60 ml/hr using a Buchler Fraction Collector (model 3R-4000). The bulk of the applied protein was collected in fractions 2-8. These fractions were pooled and assayed for enzyme activity.

b) Sephadex G-200 treatment. Gel filtration of cell-free extracts was carried out at 4°C using Sephadex G-200 as described by Andrews (1964). Before use, the columns were equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). Cell-free extract (3 ml) was applied to the top of the column by layering under a small volume of buffer already present. Pressure on the column was maintained using a LKB Perspex peristaltic pump (Model 10200) connected to the top of the column. In this way a steady flow rate of 20 ml/hr was obtained during elution. Before samples were applied to the column, uniformity of column packing was checked by passage of blue dextran 2000. Column effluent was collected in 3 ml fractions and each was assayed for protein content and serine hydroxymethyltransferase activity (this procedure is summarized in Scheme 4).

c) Ammonium sulphate fractionation at 4°C. Protein precipitating between 0-35%; 35-55%; and 55-75% saturation with $(NH_4)_2SO_4$ was collected by centrifugation at 10,000 g for 20 min. In these

SCHEME 4

Isolation of crude mitochondrial pellets by differential centrifugation


Homogenate (being ground in sucrose-AMT buffer)
500 g for 10 min
Pellet discarded
(a) supernatant
26,000 g for 20 min
(b) supernatant (soluble enzyme)
(c) Pellet
resuspended in KH_2PO_4 + 2-mercaptoethanol, pH 6.9
26,000 g for 20 min
(d) supernatant
(e) pellet
resuspended in the same solution for assay

fractionations a saturated $(NH_4)_2SO_4$ solution (pH 8.0) was employed according to the method of Green and Hughes (1955). The precipitated proteins were resuspended in saturated $(NH_4)_2SO_4$ solution and stored in the refrigerator. Samples of these fractions were also taken for enzyme assay after various periods of storage at 4°C. In such cases, the extracts were redissolved in potassium phosphate buffer at pH 8.0 followed by desalting with Sephadex G-25.

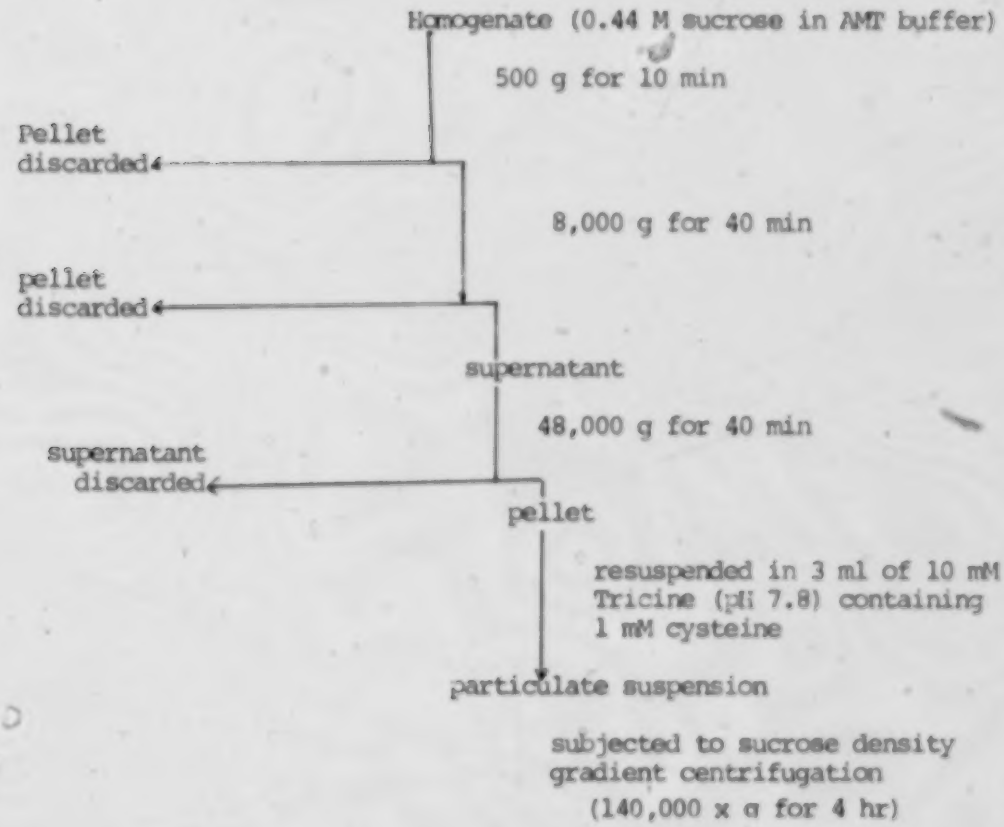
Density Gradient Centrifugation of Cell-free Extracts.

Mycelia of the formate mutant were harvested after 22 hr growth in formate-supplemented liquid medium and washed once in a medium consisting of 0.4 M sucrose, 0.17 M Tricine (pH 7.8), 10 mM KCl, 5 mM $MgCl_2$ and 4 mM dithiothreitol (Kobr *et al.*, 1969). After filtering, the mycelial pad was finely chopped with a razor blade and mixed with the 20 to 25 ml of the washing medium. After gentle grinding in a mortar, the crude extract was subjected to low speed (500 g for 10 min) centrifugation. Two further centrifugations (8,000 g for 40 min and 48,000 g for 40 min) were then applied as summarized in Scheme 5.

The final pellets were resuspended in 10 mM Tricine buffer (pH 7.8) containing 1 mM cysteine. Aliquots (1 ml) of this suspension were layered on to continuous sucrose gradients (35% to 55% w/w) contained in 9.5x1.4 cm clear centrifuge tubes. The tubes were placed in a swinging bucket SW-40 Spinco Model L2-65B ultracentrifuge. The instrument was decelerated without braking. Fractions (1 ml) were collected from the bottom of the tube at 2°C and enzyme assays were performed immediately.

SCHEME 5

Isolation of Particulate Fractions by Density Gradient Centrifugation


Homogenate (0.44 M sucrose in AMT buffer)
500 g for 10 min
Pellet discarded
8,000 g for 40 min
pellet discarded
supernatant
48,000 g for 40 min
supernatant discarded
pellet
resuspended in 3 ml of 10 mM Tricine (pH 7.8) containing 1 mM cysteine
particulate suspension
subjected to sucrose density gradient centrifugation (140,000 x g for 4 hr)

Assay of Serine Hydroxymethyltransferase Activity

a) Methylene folate production from [3-^{14}C]serine

This assay was according to Taylor and Weissbach (1965) and is based on the ready dissociation of labelled 5,10-CH_2-H_4PteGlu with carrier formaldehyde. The labelled formaldehyde is then trapped as the 5,5-dimethyl-1,3-cyclohexadione adduct and assayed for ^{14}C.

In the assay, enzyme activation was achieved by pre-incubation with 0.1 µmole pyridoxal-5-phosphate, 1.6 µmole dl-L-tetrahydrofolic acid, and 30 µmoles potassium phosphate buffer (pH 8.0) at 30°C for 5 min. The substrate, [3-^{14}C]-L-serine (1 µCi/3.5 nmole/0.1 ml of solution) was then added to initiate the reaction. The final volume of the reaction system was 0.5 ml. After incubation for 30 min at 30°C with occasional shaking, the reaction was stopped by adding in succession, 0.3 ml of 1 M Na acetate (pH 4.5); 0.2 ml of 0.1 M formaldehyde and 0.3 ml of 0.4 M dimedon dissolved in 50% ethanol. The reaction tubes were covered with parafilm and heated in a hot water bath for 5 min to complete formation of the HCHO-dimedon derivative. After cooling in an ice bath, 3 ml of toluene were added, the tubes were vigorously shaken and then centrifuged to separate toluene and aqueous phases. Samples of the upper toluene layer (0.5 ml) were removed for counting. The data are expressed in units which is the cpm incorporated in 0.5 ml of toluene. Control systems in which 0.1 ml of potassium phosphate buffer (pH 8) replaced the enzyme were included in all assays.

b) Serine production from [2-^{14}C]glycine

The cytoplasmic and mitochondrial fractions of the wild type and *for* mutant were used in these assays. Enzyme activation was achieved by pre-incubation with 0.1 µmoles pyridoxal-5-phosphate, 1.6 µmoles dl-L-tetrahydrofolic acid, 15 µmoles potassium phosphate buffer (pH 8.0) and 2 µmoles formaldehyde for 5 min at 30°C. After adding 0.1 µmoles of

glycine (0.5 μCi of 2-^{14}C), the reaction system was mixed and incubation at 30°C continued for 30 min. The control lacked formaldehyde. Enzyme activity was terminated by immersion in a boiling water bath for 5 min. After cooling in an ice bath and centrifugation to remove denatured protein, amino acids were recovered by passage through Dowex 50W (X8; H^+ form). The resin was first washed with 30 ml of distilled water, followed by 40 ml of 6N HCl to elute glycine and serine. The acid effluent was dried *in vacuo* at 40°C and the residue was redissolved in 0.2 M sodium citrate buffer (pH 2.2). Serine and glycine were then separated using an amino acid analyzer (Beckman Instruments, Model 121) as described by Clandinin and Cossins (1972). The data are expressed in units which is the cpm incorporated in the reaction system.

Assays of Isocitrate Lyase Activity

The enzyme was assayed according to Dixon and Kornberg (1959). The mycelium (20 mg), after 22 hr's growth, was suspended in (30 ml) 0.2 M phosphate buffer (pH 6.0) containing 0.5 mM cysteine, 1 mM EDTA and 0.1 of a ml of polyoxyethylene (20) sorbitan monooleate, a detergent (Kobr *et al.*, 1965). Homogenization was achieved by grinding in a mortar with fine sea sand. The homogenate was centrifuged at 16,000 x g for 20 min to remove cellular debris. Extracts of the wild type and *for* mutant were then desalted by passage through columns of Sephadex G-25 as described earlier.

The lyase assay involved measurement of the absorbance increase at 324 nm due to formation of glyoxylic acid phenylhydrazone. The reaction system contained in 3 ml, 0.05 M imidazole buffer (pH 7.2); 3.9 mM $MgCl_2$; 3.9 mM cysteine HCl (pH 7.2); 2.6 mM phenylhydrazine (pH

7.2); 7.7 mM L-isocitrate; and enzyme extract. The reaction was started by addition of isocitric acid and the controls lacked this substrate. The reaction was carried out in quartz curvettes maintained at 30°C and absorbance changes at 324 nm were followed for approximately 20 min. A standard curve was constructed using various concentrations of sodium glyoxylate under these incubation conditions. Enzyme activities are expressed in µmoles of glyoxylate produced/mg protein/hr.

Assay of L-alanine-glyoxylate Aminotransferase Activity

The assay was according to Combepine (1969). Mycelia of 20 mg fresh weight were ground with sand in (30 ml) 50 mMphosphate buffer (pH 7.4); 0.14 M NaCl; 100 mM cysteine-HCl; 1 mM EDTA and 0.05% polyoxyethylene (20) sorbitan monooleate using a pestle and mortar. The homogenate was then centrifuged at 4,500 g for 10 min at 3°C and the supernatant was immediately desalted by passage through a column of Sephadex G-25.

In the assay, the enzyme extract (0.25 ml) was pre-incubated for 5 min at 30°C with 0.2 M Tris buffer (pH 8.2) containing 2.5 µmoles of $MgCl_2 \cdot 6H_2O$, 12.5 µmoles of L-alanine and 0.01 mg pyridoxal-5-phosphate. After the addition of 12.5 µmoles glyoxylate (0.05 µCi [1-^{14}C]-glyoxylate, the reaction system, total volume (0.55 ml) was incubated for periods of 5 and 10 min at 30°C. The control lacked enzyme, this being replaced by the Tris buffer (pH 8.2). The reaction was stopped by heating in a hot water bath for 5 min, followed by cooling in an ice bath. Glycine was recovered with the aid of an amino acid analyzer following prior fractionation on Dowex 50W (X8; H^+ form) as mentioned earlier (page 27). Aminotransferase activity is expressed as cpm incorporated/mg enzyme protein.

Assay of Malate Synthetase Activity

The assay was that used by Combepine (1969). Mycelia of 20 mg fresh weight were ground with sand in a medium containing: 0.4 M sucrose, 0.17 M Tricine (pH 7.8), 10 mM KCl, 5 mM $MgCl_2$ and 4 mM dithiothreitol. Subsequent fractionation of the homogenate included a low speed centrifugation (500 g for 10 min). The supernatant containing the enzyme extract was eluted with the above buffer and was desalted by passage through a column of Sephadex G-25.

The enzyme was pre-incubated for 5 min at 30°C with 0.12 M Tris buffer (pH 8.1); 5 mM $MgCl_2$ and 2.5 mg acetyl-CoA. The reaction was initiated by addition of 15.2 mM glyoxylate (pH 8.1). Final reaction volumes were 3 ml. After a reaction time of 10 min at 30°C, the reaction was stopped by addition of 0.12 mM dithiobis. After a further 5 min, absorbancies of the tubes were taken at 412 nm. The controls contained no enzyme extract. In both the controls and the reaction systems, absorbancies were read against distilled water.

Succinic Dehydrogenase Activity

The enzyme was assayed by the colorimetric method of Hiatt (1961). This method follows the rate of reduction of sodium 2,6-dichlorophenolindophenol with N-methyl phenazonium sulphate acting as the final electron acceptor. The complete assay system (3 ml) contained 0.05 M potassium phosphate buffer (pH 7.4), 0.04 M sodium succinate, 0.01 M potassium cyanide, 0.03 mM sodium 2,6-dichlorophenol-indophenol, 0.3 mg/ml N-methyl phenazonium sulphate and enzyme. Enzyme activity was insignificant when succinate and N-methyl phenazonium sulphate were omitted. Decreases in absorbance were monitored at 600 nm. One unit of enzyme activity was taken as the amount of enzyme causing a decrease

in absorbancy of 0.01 per min.

Glyoxylate [1-^{14}C] Feeding Experiments

Mycelia of both strains were cultured at 25°C for 22 hr as described earlier. Samples (25 ml) of these cultures were then harvested and resuspended in 25 ml of fresh medium containing 3 µCi of sodium glyoxylate [1-^{14}C]. After incubation periods of 5, 15 and 30 min at 25°C with shaking, the feeding periods were terminated by addition of 25 ml of ethanol. The samples were then boiled for 15 min in a hot water bath. After grinding and centrifugation (500 g) the whole extracts were applied to columns of Dowex 50W (X8; H^+ form) (Canvin and Beevers, 1961). The column (1x12 cm) was washed with 30 ml of water, followed by 40 ml of 6N NCl which eluted the amino acids. The water wash from the above columns was applied to anion exchange resin, Dowex 1-X8 in the formate form, for separation of sugars and organic acids (Canvin and Beevers, 1961).

Individual organic acids were separated by paper chromatography using Whatman No. 1 paper with butanol/glacial acidic acid/water (4:1:5) as solvent system. The papers were run (descending solvent) for 40 hr at room temperature. After drying, 1 cm-square strips were cut and counts were obtained with a scintillation counter.

Free Amino Acid Determinations

The amino acids of most interest in the present work were glycine and serine and these were determined quantitatively using an automatic amino acid analyzer. After various enzyme incubations (see earlier), the reaction systems were centrifuged to remove denatured protein. The supernatants were then passed through columns of Dowex

50W-X8 (H^+ form) 100-200 mesh (Bio Rad Laboratories, Richmond, California, U.S.A.) to absorb the amino acids. These were eluted by addition of 40 ml of 6N HCl which was subsequently removed by drying *in vacuo*. The dried residue was dissolved in 3 ml of 0.2 M citrate buffer (pH 2.2) and 0.5 ml samples were analyzed in the amino acid analyzer (Clandinin and Cossins, 1972). Radioactivity of effluents from the analyzer were determined by scintillation counting and amino acid pools were determined by reaction with ninhydrin.

Estimation of Protein Contents

The protein contents of various enzyme preparations were estimated colorimetrically as described by Lowry *et al.* (1951). Crystalline egg albumin was used as a reference standard.

RESULTS

Effect of Media Supplements on Growth of the Formate Mutant

Harrold and Fling (1951) observed that a mutant strain (C-24) of *Neurospora crassa* only grew on minimal medium when supplemented with formate. The workers showed that this mutant also grew well on adenine or on adenine plus methionine. Growth was poor in media containing glycine and serine, the latter having no effect when the mutant was grown suboptimally on formate or adenine. Cossins *et al.* (1976) studied the effect of media supplements on growth of this mutant in more detail. They showed that the mutant grew well in Vogel's minimal medium supplemented with 10 mM formate. The strain did not grow as well when adenine, L-methionine, L-serine, glycine or glyoxylate were added separately to the medium.

The present studies have used the same culture methods with freshly harvested conidia being used to inoculate samples of defined liquid medium. Mycelial production was exponential within 18 hr and samples were routinely harvested after 22 hr of growth.

Serine Hydroxymethyltransferase Activities of the Wild Type and *for* Mutant

Figure 1 shows the effect of pH on the activity of serine hydroxymethyltransferase extraction from the wild type and mutant. In both cases, maximal activity occurred at approximately pH 8.0. All subsequent assays were carried out at this pH value. The optimum pH for these *N. crassa* enzymes is different from that (pH 8.5) reported by Botsford and Parks (1969) for

FIGURE 1

The Effect of pH on Serine Hydroxymethyltransferase Activity

Assays of wild type soluble enzyme and *for* mutant particulate enzyme were made in reaction mixtures having a total volume of 0.5 ml. Both systems contained the following:

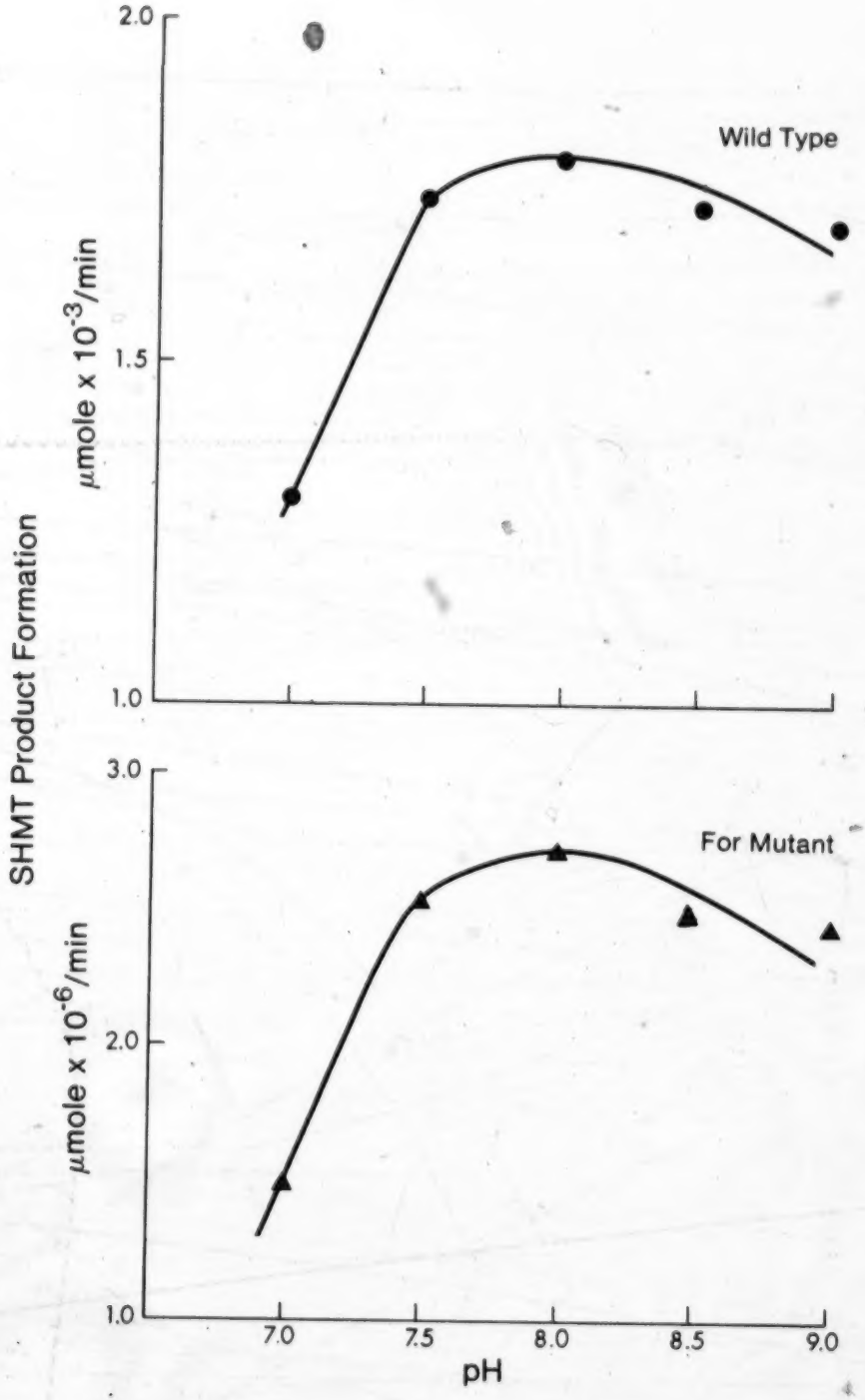
0.1 µmole pyridoxal-5-phosphate; 1.6 µmole dl-L-tetrahydrofolic acid; and 30 µmoles potassium phosphate buffer adjusted to the pH values indicated.

For wild type soluble enzyme assay the following were added in addition: L-[3-^{14}C]serine (1 µCi/3.5 mmol/0.1 ml solution) and 120 µg of 26,000 g supernatant protein.

For the *for* mutant particulate enzyme assay the reaction system contained:

2 µmoles formaldehyde; 0.1 µmole glycine, containing 0.01 µCi 2-^{14}C, and mitochondrial protein.

Both assays were incubated for 30 min at 30^{0}C with shaking. The reactions were terminated as described in the Materials and Methods section. The data are average values from two separate experiments, each assayed in duplicate. The range in data obtained at pH 8 was 1.81 to 1.82 x 10^{-3} µmoles/min for the wild type and 2.65 to 2.70 x 10^{-6} µmoles/min for the mutant.


SHMT Product Formation
2.0
1.5
1.0
μmole x 10-3/min
Wild Type
3.0
2.0
1.0
μmole x 10-6/min
For Mutant
7.0
7.5
8.0
8.5
9.0
pH

S. cerevisiae and is significantly different from the 6.8-7.6 optimum range detected in *Plasmodium* by Platzer and Campuzano (1976).

In a study of the effect of incubation time on product formation using the Taylor and Weissbach (1965) assay procedure, the rate of 5,5-dimethyl-1,3-cyclohexadione adduct formation was a linear function of time for approximately 15 min (Fig. 2). An identical period has been noted by Botsford and Parks (1969) in their studies of the yeast enzyme. However, in assays of the reverse reaction, particularly with the particulate enzyme of the *for* mutant, appreciable serine formation still occurred at 30 min. In such assays reaction times of 30 min were employed.

Attempts were made to purify the enzyme by $(NH_4)_2SO_4$ precipitation and to check its stability on storage. The fractionation procedure is described in the Materials and Methods section. In a typical experiment, the initial specific enzyme activity was 8.5×10^{-4} µmole/hr/mg protein. Table 1 shows that specific activities increased from this value following $(NH_4)_2SO_4$ precipitation. The increase was approximately 8 and 200 fold for the 35-55% and 55-75% fraction, respectively. These fractions were examined for stability following periods of storage at 4°C. Enzyme activity was labile under such conditions as only 50% of the activity remained after 48 hr of storage. Further purifications were not attempted and all subsequent assays were made with freshly prepared mycelial extracts. However, it was found that when the 55-75% fraction was passed through a column of Sephadex G-25, the specific enzyme activity increased by a factor of 55 (Table 2). Conceivably a low molecular weight inhibitor of enzyme activity was removed by this treatment.

FIGURE 2

The Relationship Between Serine Hydroxymethyltransferase Activity and Reaction Time

Reaction mixtures containing the following in a total volume of 0.5 ml:

0.1 μmole pyridoxal-5-phosphate; 1.6 μmole dl-L-tetrahydrofolic acid; 30 μmoles potassium phosphate buffer, pH 8.0; L-[3-^{14}C]serine of radioactivity of 1 μCi/3.5 mmol per 0.1 ml of solution and the cell-free extract (26,000 g) enzyme protein of the wild type.

After 5 min of pre-incubation the reaction systems were incubated at 30°C for the various times indicated. The assay was as outlined in the Materials and Methods section.

The data are average values from two separate experiments.

SHMT (μmole product x 10^{-3})
0
2
4
6
8
10
0
5
10
15
20
25
Minutes

TABLE 1

Loss of Serine Hydroxymethyltransferase Activity after $(NH_4)_2SO_4$ Precipitation on Storage

Days after extraction	Specific enzyme activities of serine hydroxymethyltransferase (μmole x 10^{-4}/hr/mg protein)	
	35-55% fraction After $(NH_4)_2SO_4$ precipitation	55-75% fraction after $(NH_4)_2SO_4$ precipitation
Initial	72.0	2280
2	38.4	1200
4	3.6	114

Cell-free extract was prepared as described in the Materials and Methods section. Protein precipitating at 35-55% and 55-75% saturation with $(NH_4)_2SO_4$ was collected by centrifuging at 10,000 g for 20 min. In these fractionations a saturated $(NH_4)_2SO_4$ solution was employed according to the method of Green and Hughes (1955).

The enzyme assay was carried out on these fractionations after various periods of storage at 4°C as indicated. The data are average values from duplicate experiments.

TABLE 2

Specific Enzyme Activities of Serine Hydroxymethyltransferase after Desalting with Sephadex G-25

% Fraction after $(NH_4)_2SO_4$ precipitation	Specific enzyme activities of serine hydroxymethyltransferase (μmole x 10^{-4}/hr/mg protein		Ratio of Specific activities (initial/final)
	Before Sephadex G-25	After Sephadex G-25	
55-75	410	2280	55

Cell-free extract was prepared from mycelia grown for 22 hr as described in the Materials and Methods section. Protein precipitating at 55-75% saturation with $(NH_4)_2SO_4$ was collected by centrifuging at 10,000 g for 20 min. After redissolving in buffer the protein extract was passed through a column of Sephadex G-25. The enzyme was assayed as described in the Materials and Methods section. The data represent the average of two separate experiments.

In further enzyme studies, the production of 5,5-dimethyl-1, 3-cyclohexadione adduct in the Taylor and Weissbach (1965) assay was compared in the wild type and *for* mutant. The experimental procedure for isolation of the various fractions is summarized in Scheme 5. For comparative purposes data are expressed in units/mg dry wt of mycelium and in units/µg protein (Table 3).

In the wild type, a soluble enzyme, recovered in the 26,000 g supernatant, accounted for the bulk of recovered activity (Table 3A). In the *for* mutant neither the 26,000 g supernatant nor the 26,000 g mitochondrial pellet, after resuspension, displayed appreciable production of 5,5-dimethyl-1,3-cyclohexadione adduct from serine (Table 3B). In fact, the data suggests that the soluble enzyme was partially or completely lacking in the mutant. However, it might be argued that the soluble enzyme in the mutant favours the formation of serine from glycine. To examine this possibility, experiments on the interconversion of glycine and serine in both wild type and *for* mutant were carried out.

In these experiments, equal specific radioactivities of serine and glycine were used as substrates in assays described in the Materials and Methods section. The cytoplasmic and mitochondrial extracts of the wild type and *for* mutant were prepared by differential centrifugation. The data (Table 4) suggest that the soluble enzyme of the wild type favours the formation of glycine from serine. However, mitochondrial extracts of the mutant favoured the reverse reaction. The soluble enzyme in the mutant had slightly higher activity in the glycine to serine reaction but this could have been due to some leakage of the particulate enzyme during preparation.

TABLE 3

Production of Glycine from Serine by Various Cellular Fractions of *N. crassa*

A. Wild Type Cellular fractions	units/mg dry wt.	units/µg protein
500 g supernatant (a)	3308	37.5
26,000 g supernatant (b) (soluble enzyme)	3670	40.4
26,000 g pellet (c)	577	6.5
26,000 g supernatant (d) after resuspension	869	9.8
26,000 g pellet (e) after resuspension	401	2.1

B. *for* Mutant Cellular fractions	units/mg dry wt.	units/µg protein
500 g supernatant (a)	29	0.30
26,000 g supernatant (b) (soluble enzyme)	30	0.31
26,000 g pellet (c)	29	0.26
26,000 g supernatant (d) after resuspension	27	0.24
26,000 g pellet (e) after resuspension	26	0.16

The experimental procedure is outlined in Scheme 4. The enzyme was assayed as described in the Materials and Methods section. The data are average values from duplicate experiments. Letters a-e refer to steps in the centrifugation procedure summarized in Scheme 4.

For definition of units see Page 27.

TABLE 4

Interconversion of Glycine and Serine: *N. crassa* Wild Type and *for* Mutant

Strain	units/mg dry wt		units/μg protein	
	Ser → Gly	Gly → Ser	Ser → Gly	Gly → Ser
Wild type				
soluble	3670	826	40.4	7.4
particulate	401	1980	2.1	17.5
for mutant				
soluble	30	980	0.31	10.0
particulate	26	5011	0.16	38.0

Equal specific radioactivities of serine and glycine (0.5 μCi) were used as substrates. The assay method is described in the Materials and Methods section. The soluble and mitochondrial enzyme extracts for both wild type and *for* mutant were contained in 26,000 g supernatant and 26,000 g pellet after resuspension. This was outlined in Scheme 4. The data are average values from duplicate experiments. For definition of units see Pages 27 and 28.

At this stage it was of some interest to determine whether activity in the 26,000 g pellet was associated with the mitochondria.

After isopycnic centrifugation of the 28,000 g particulate material, a band of mitochondria was detected (Fig. 3). This band, located principally in fraction 8, contained a clear peak of succinic dehydrogenase activity. As this dehydrogenase is exclusively mitochondrial (Combepine *et al.*, 1969), it follows that serine hydroxymethyltransferase activity of the *for* mutant (Fig. 3) is also localized in this organelle. Succinic dehydrogenase in this work was associated with similar sucrose densities as reported earlier by Combepine *et al.* (1969). It was also indicated by Combepine *et al.* (1969) that the density distribution of enzyme markers of glyoxylate cycle, isocitrate lyase and malate synthetase is significantly different from that of the tricarboxylic acid cycle.

In further studies of the enzyme, Sephadex G-200 was employed to determine whether the soluble enzyme of the wild type and the mitochondrial enzyme in the *for* mutant might differ in molecular weight. For such experiments, enzyme extracts were obtained by differential centrifugation. A single, sharp peak of blue dextran indicated that column packing was uniform (Fig. 4). Enzyme assays showed that soluble activity from the wild type and mitochondrial activity from the *for* mutant were recovered in different column fractions (Fig. 4). The soluble enzyme was collected in fractions 25 to 32 with peak activity in fraction 27. The mitochondrial enzyme of the mutant was, however, collected in fractions 30 to 35 with the peak activity in fraction 32. These assays were made with [3-^{14}C]serine as substrate, consequently activity for the mitochondrial enzyme was low.

FIGURE 3

Density Gradient Centrifugation of Particulate

Serine Hydroxymethyltransferase

The enzyme assay of succinic dehydrogenase on (1 ml) fractions contained the following in a total volume of 3 ml:

0.05 M potassium phosphate buffer (pH 7.4); 0.04 M sodium succinate; 0.01 M potassium cyanide; 0.03 mM sodium 2,6-dicnloro-phenol-indophenol; 0.3 mg/ml N-methylphenozonium sulphate and the enzyme protein.

The decrease in absorbance was taken at 600 nm.

The enzyme assay of serine hydroxymethyltransferase isoenzyme in the *for* mutant was carried out on (1 ml) fractions and was described in the Materials and Methods section.

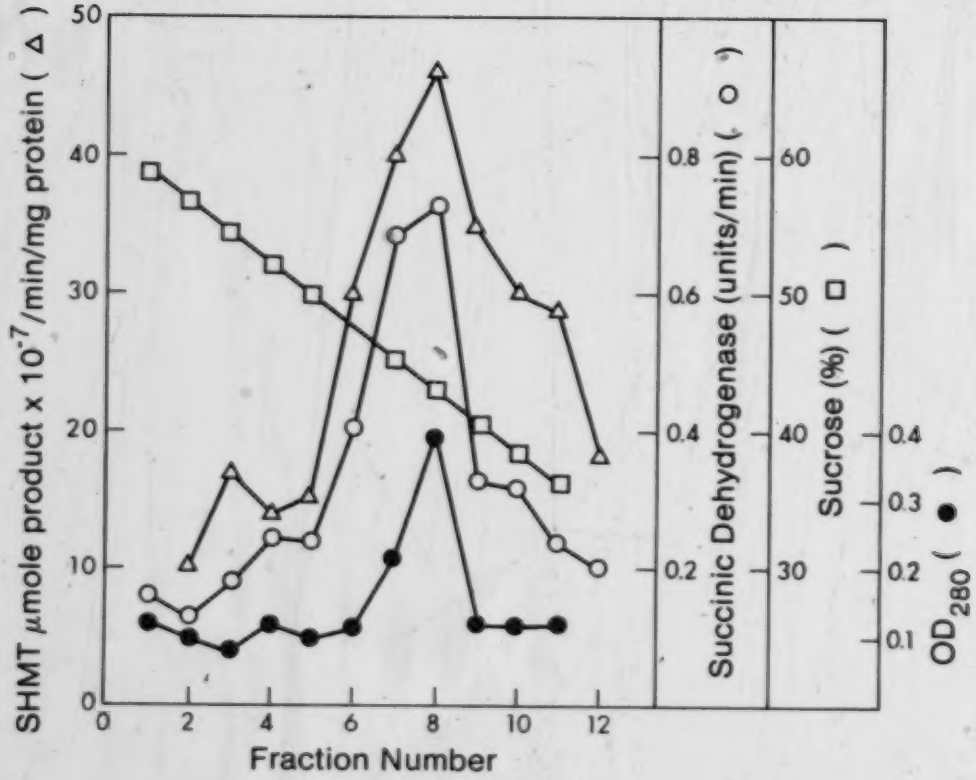

SHMT μmole product x 10⁻⁷/min/mg protein (Δ)
Succinic Dehydrogenase (units/min) (○)
Sucrose (%) (□)
OD280 (●)
Fraction Number
0
10
20
30
40
50
0.2
0.4
0.6
0.8
30
40
50
60
0.1
0.2
0.3
0.4
0
2
4
6
8
10
12

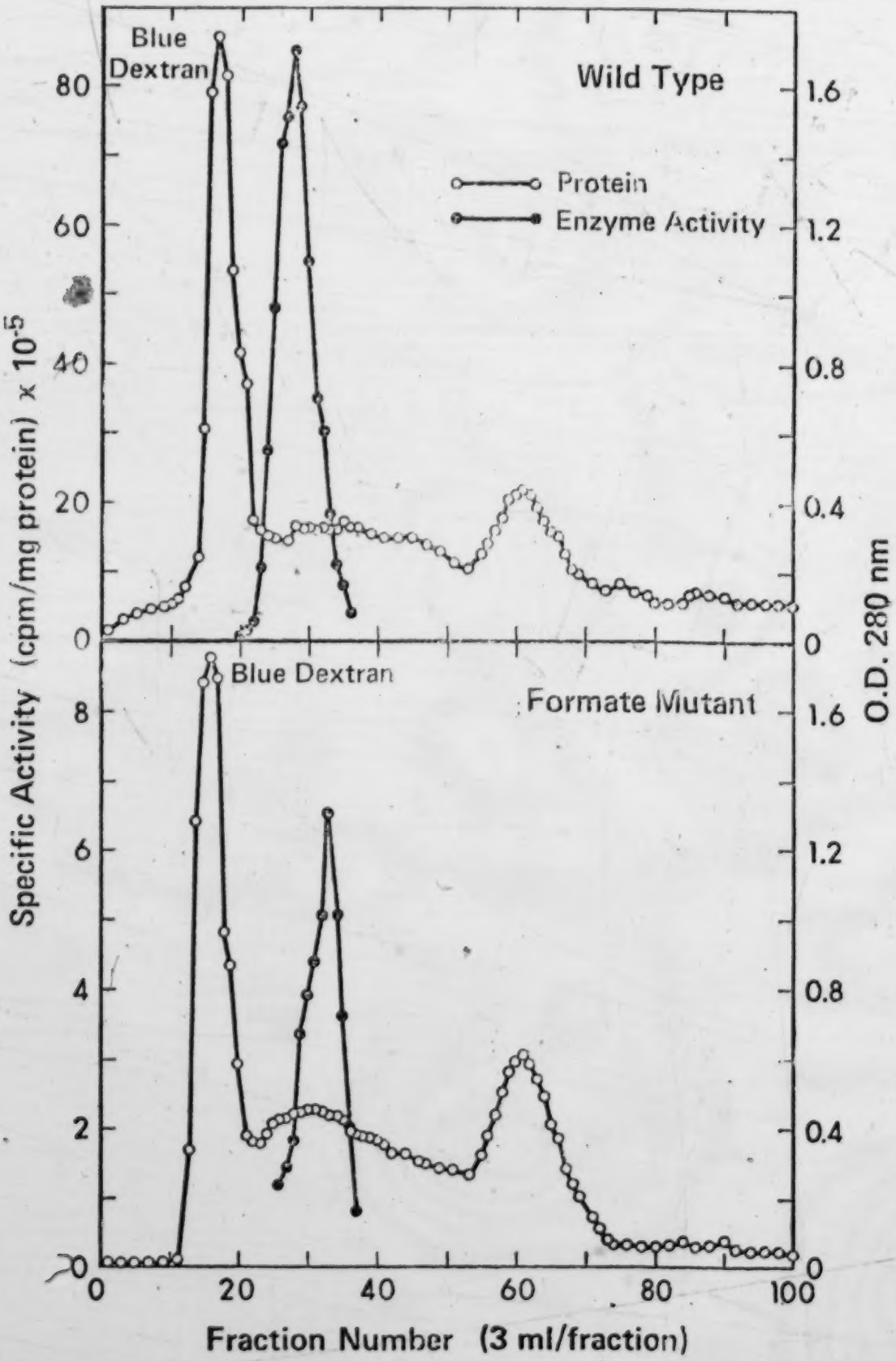
FIGURE 4

Gel Filtration of Serine Hydroxymethyltransferase

A. The mycelia of the wild type was cultured in minimal Vogel's liquid medium for 22 hr at 25°C. The protein extract was prepared after centrifugation at 500 g.

B. The mycelia of the *for* mutant was cultured under the same conditions except the Vogel's medium was supplemented with 10^{-2}M sodium formate for the growth of the mutant. The crude mitochondrial pellet was prepared after centrifugation at 26,000 g.

Samples of protein extract were applied to the column and eluted with potassium phosphate buffer (pH 8.0). Fractions of 3 ml were collected.


Blue Dextran
Wild Type
Protein
Enzyme Activity
Blue Dextran
Formate Mutant
Specific Activity (cpm/mg protein) x 10^{-5}
O.D. 280 nm
Fraction Number (3 ml/fraction)
0
20
40
60
80
100
2
4
6
8
0.4
0.8
1.2
1.6

The Presence of Glyoxylate Cycle Enzymes in the Wild Type and Mutant

A. Isocitrate lyase activity

The pH and substrate concentration for the assay of isocitrate lyase followed that of Kobr *et al.* (1965). The present work, however, checked both parameters over a pH range of 6.2 to 9.2 and over an isocitrate concentration range of 6.6 mM to 8.8 mM. The optimum values obtained, namely a pH optimum of 7.2 and a saturating isocitrate concentration of 7.7 mM, were the same as described by Kobr *et al.* (1965).

The assay method has been described in the Materials and Methods section and followed that of Dixon and Kornberg (1959). Foo *et al.* (1971) examined the regulation of isocitrate lyase in autotrophic and photoheterotrophic cultures of *Gloeomonas* species. They found that the enzyme from CO_2 or acetate-grown cells had an optimum of pH 7.3-7.5.

Figure 5 shows the activities of isocitrate lyase in terms of absorbances at 324 nm. From the slope of each curve it was calculated that the specific enzyme activities were 0.61 µmole/mg protein/hr and 1.56 µmole/mg protein/hr for the wild type and *for* mutant, respectively. As shown in Figure 5, a conspicious lag period occurred before product formation was detected. This is commonly found in assays of isocitrate lyases (Dixon and Kornberg, 1959). In each case (Fig. 5) velocities were calculated from the lines shown.. A comparison of the reaction rates shows that glyoxylate formation was some two and a half times greater in the mutant.

FIGURE 5

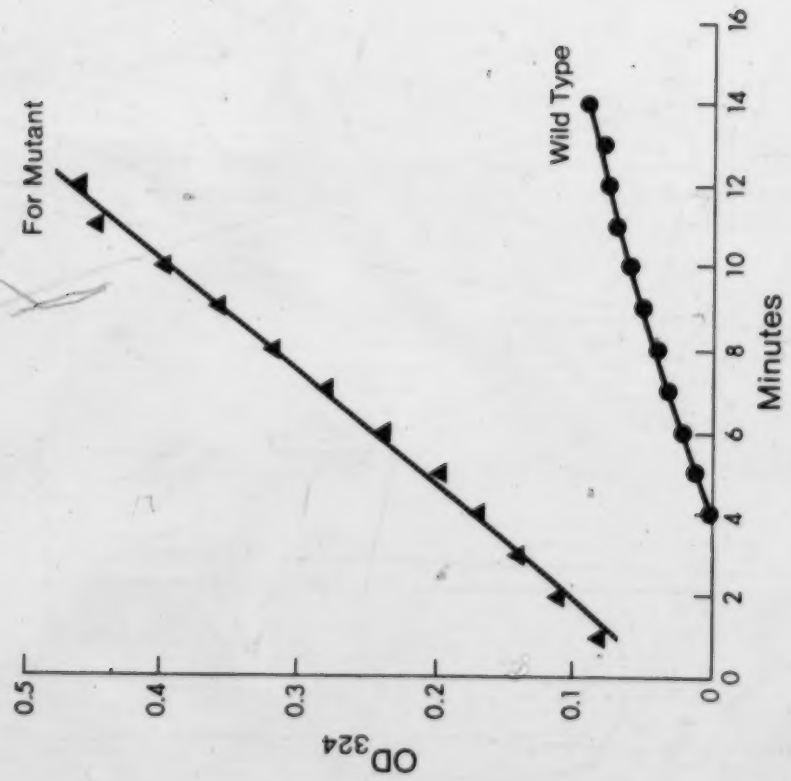
Isocitrate Lyase Activity in *N. crassa* Wild Type and *for* Mutant

Wild type enzyme reaction mixtures contained the following in a total volume of 3 ml:

0.05 M imidazole buffer (pH 7.2); 3.9 mM $MgCl_2$; 3.9 mM cysteine HCl (adjusted to pH 7.2); 2.6 mM phenylhydrazine (pH 7.2); 7.7 mM L-isocitrate; enzyme extract and water. The control contained no L-isocitric acid.

Changes in absorbance at 324 nm were followed throughout the reaction.

For mutant enzyme activity was as above.


For Mutant
Wild Type
OD_{324}
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
Minutes

B. L-alanine:glyoxylate transaminase activity

The reaction was followed over the incubation periods summarized in Figure 6. The time course curves for the wild type and *for* mutant are similar with net product formation occurring for approximately 15 min. Cossins and Sinha (1965), working on the rate of glycine formation in higher plant systems, reported an optimum incubation time of 30 min for pea leaf extracts. Calculation of initial reaction velocities showed that the *for* mutant had approximately twice the transaminase activity of the wild type.

C. Malate synthetase activity

In these assays, the control cuvette lacked enzyme. The reaction was followed at 412 nm according to the equations:

1 acetyl-CoA + 1 glyoxylate → 1 malate + 1 CoA

1 CoA + 1 DNTB → 1 CoA complex + 1 anion nitrobenzoic thiol liberated

The data in Table 5 are for different assays and have been corrected for absorbance changes occurring in the control cuvettes. It is clear that the wild type contained higher levels of malate synthetase than the mutant.

Glyoxylate Feeding Experiments

As the mutant contained more ability to produce glyoxylate and to convert this compound into glycine *in vitro*, further experiments were carried out with growing cultures to assess possible differences in the metabolism of added [1-^{14}C]glyoxylate.

The procedure for supplying the labelled substrate is described in the Materials and Methods section. The data in Table 6 are expressed in cpm/μmole and cpm/mg dry wt. It is clear from these data

FIGURE 6

Glycine Production by L-Alanine:Glyoxylate Transaminase in *N. crassa* Wild Type and *for* Mutant

Wild Type Enzyme: The reaction mixtures contained the following in a Total volume of 0.55 ml:

0.2 ml (0.2 M) Tris buffer (pH. 8.2); $MgCl_2 \cdot 6H_2O$ (2.5 µmoles); L-alanine (12.5 µmoles); 0.01 mg of pyridoxal-5-phosphate; 12.49 µmoles glyoxylate containing 0.05 µCi 1-^{14}C, and mycelial extract (274 µg protein).

For Mutant: Enzyme assay was as above. The reaction system contained 220 µg of protein.

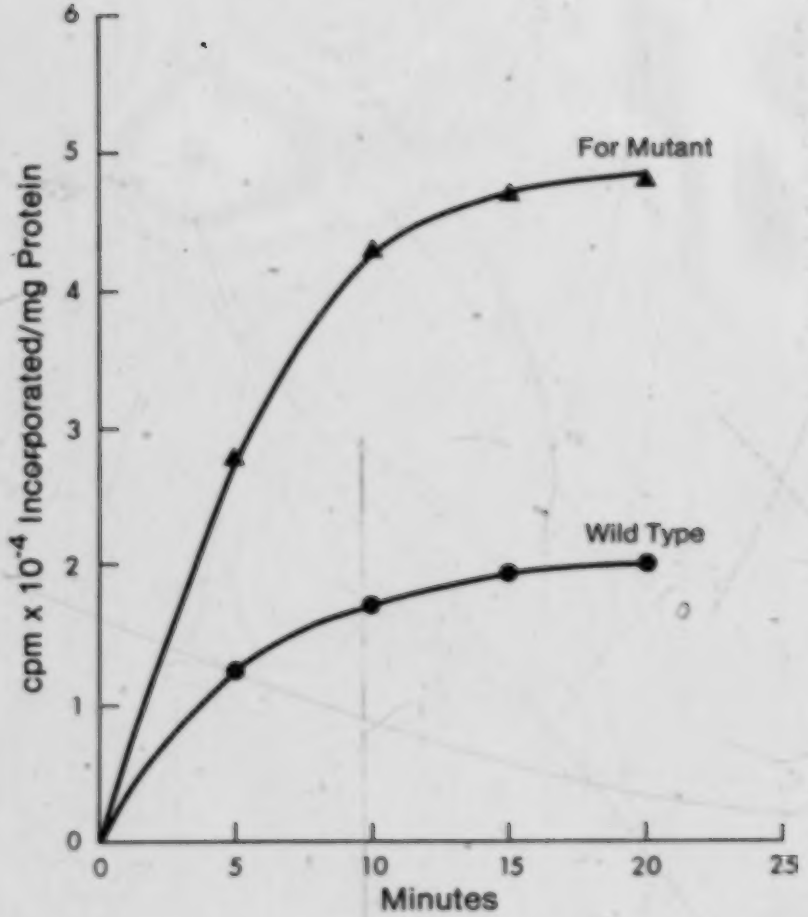

For Mutant
Wild Type
cpm x 10⁻⁴ Incorporated/mg Protein
Minutes
0
1
2
3
4
5
6
0
5
10
15
20
25

TABLE 5

Malate Synthetase Activity: *N. crassa* Wild Type and *for* Mutant

Strain	Absorbances 5 min	10 min	15 min	Average absorbance at 10 min	Δ absorbance per min
Wild type	0.20, 0.21	0.43, 0.43	0.47	0.43	0.043
for mutant	0.15, 0.15	0.31, 0.315	0.34	0.31	0.031

Mycelia were grown for 22 hr for both the wild type and *for* mutant. Sephadex G-25 treated extract was prepared as described in the Materials and Methods section. The enzyme assay was outlined in the above section. The absorbancies were taken at 412 mμ after addition of 0.12 mM dithiobis for 5 min. In assays of both strains the reaction system contained 183 μg of protein.

TABLE 6

Incorporation of [1-^{14}C]Glyoxylate by *Neurospora crassa*, Wild Type and *for* Mutant: Principal Products

Free Amino Acid	Time (min)	Wild Type			*for* Mutant		
		5	15	30	5	15	30
Glycine		**35.1** *(410.4)*	**222.7** *(333.1)*	**164** *(284)*	**157.7** *(626.4)*	**1348.5** *(1078.8)*	**1980.8** *(2971.2)*
Serine		**29.3** *(233.2)*	**84** *(150)*	**129** *(241)*	**63.8** *(319.2)*	**108.5** *(260.4)*	**157.1** *(518.4)*
Aspartic acid		**87.4** *(583)*	**537.1** *(712)*	**712.5** *(1065)*	**48.6** *(333.6)*	**216.5** *(554.4)*	**277.0** *(698)*
Protein amino acids							
- Glycine		**0.13** *(56)*	**0.35** *(170)*	**1.09** *(340)*	**0.85** *(53)*	**1.5** *(200)*	**2.1** *(534)*
- Serine		**0.13** *(28)*	**0.24** *(72)*	**0.59** *(112)*	**0.50** *(19)*	**0.8** *(115)*	**0.9** *(260)*
Organic acids		*(35)*	*(49)*	*(103)*	*(28)*	*(37)*	*(45)*
Sugars		*(42)*	*(67)*	*(81)*	*(48)*	*(75)*	*(105)*

Mycelia were grown in Vogel's medium, supplemented with formate for the mutant. After harvesting and resuspension in the growth media, 25 ml samples were incubated with 3 µCi of [1-^{14}C]glyoxylate. The cultures were incubated at 25°C with shaking. The[1-^{14}C]glyoxylate feeding was terminated after 5 min, 15 min, and 30 min time intervals.

The figures in bold type, for free amino acids and protein amino acids, are expressed in cpm x 10^{-2}/µmole. The figures in italics, for free amino acids and protein amino acids, are expressed in cpm/mg dry wt; for organic acids and sugars are expressed in cpm x 10^{-3}/mg dry wt.

that glycine synthesis in the *for* mutant was considerably greater than that observed in the wild type. Furthermore, serine was also more heavily labelled in the mutant. In contrast, greater amounts of glyoxylate carbon entered the aspartate pool of the wild type, although this amino acid was also an important product in the mutant. The data, therefore, support the contention that glycine can arise from glyoxylate in *Neurospora*.

DISCUSSION

General Properties and Localization of Serine Hydroxymethyltransferase

Serine hydroxymethyltransferase has been found in a number of higher plants and in a variety of plant tissues. The most complete details of the properties of the enzyme have been obtained in studies with corn seedlings (Hauschild, 1959), wheat leaves (Cossins and Sinha, 1964), tobacco root *Nicotiana rustica* (Pratner and Sisler, 1966), and cauliflower (Mazelis and Liu, 1967). The pH optima for enzyme activity from these sources have been found to be 6.6, 7.6, 4.0 and 7.5, respectively. In *Neurospora crassa* the pH optima is 8.0 (Fig. 1). In many studies, the enzyme has a requirement for pyridoxal phosphate. This has been demonstrated for the enzyme from cauliflower, (*Brassica oleracea* var. *botrytis* L), (Mazelis and Liu, 1967), corn (Hauschild, 1959), and turnip (Wilkinson and Davies, 1958). The enzyme from wheat leaves (Cossins and Sinha, 1964) did not appear to require added pyridoxal phosphate for activity and that from *Nicotiana rustica* showed no response to this coenzyme until treated with KCN or semicarbazide. (Pratner and Sisler, 1966). In most studies the enzyme had an absolute requirement for tetranydrofolic acid and displayed narrow specificity for L-serine.

Metal ions have been found to have marked effects on the activity of the tobacco and cauliflower enzymes. The tobacco enzyme is activated by magnesium and calcium ions at 0.001 M concentration with the former being most effective (Prather and Sisler, 1966). Sodium and potassium ions also gave some activation at 0.05-0.10 M. The cauliflower enzyme is also stimulated by K^+ but other metal ions such as Na^+, Mg^{2+}

or Ca^{2+} had very little effect (Mazelis and Liu, 1967).

Serine hydroxymethyltransferase apparently contains a sulfhydryl group necessary for activity since the usual sulfhydryl inhibitors such as iodacetic acid and p-chloromercuribenzoic acid inhibit the enzyme reaction (Prather and Sisler, 1966). In common with other separations, the serine hydroxymethyltransferase of *Neurospora crassa* can be readily fractionated with $(NH_4)_2SO_4$ and is partially purified by chromatography on Sephadex G-200 (Tables 1 and 2, Fig. 4).

There is now abundant evidence that the enzyme has importance in the interconversion of serine and glycine. This role was first suggested by Shemin (1964) who found that when ^{15}N, ^{13}C-serine was fed to rats and guinea pigs along with benzoate, the ^{13}C was incorporated with little dilution into the carbonyl carbon of hippuric acid. Other evidence was provided by examination of liver protein and by work with intact rats (Byerrum *et al.*, 1955; Alexander and Greenberg, 1956; Goldsworthy *et al.*, 1949; Sakami, 1948; Siekevitz and Greenberg, 1949; Winnick *et al.*, 1948).

Two forms of serine hydroxymethyltransferase are present in *Saccharomyces cerevisiae*, rabbit liver, rat liver and possibly also in cauliflower (*Brassica oleracea* var. *botrytis* L.) (Zelikson and Luzzati, 1976; Fujioka, 1969; Nakano *et al.*, 1968; Mazelis and Liu, 1967). The isoenzymes purified from these various sources are clearly associated with soluble and mitochondrial fractions. This is also the case in *N. crassa* (Table 4, Figs. 3, 4). In some tissues such as rabbit liver, the isoenzymes had similar physical properties but in other cases the isoenzymes differ, a situation observed by Nakano *et al.* (1968) for rat liver. A thymidylate-less mutant of *S. cerevisiae* contains only a

soluble serine hydroxymethyltransferase (Zelikson and Luzzati, 1976). The lack of the mitochondrial form was accompanied by a general requirement for dTMP, methionine, adenine and histidine (Zelikson and Luzzati, 1976). This suggests that the two isoenzymes may have different metabolic functions with the mitochondrial isoenzyme having importance in supplying methylenetetrahydrofolate for further synthesis of one-carbon metabolites which are presumably compartmented in yeast.

In *N. crassa*, the *for* mutant is clearly deficient in the soluble isoenzyme. Furthermore, the mitochondrial isoenzyme appeared to favour the formation of serine from glycine (Tables 3, 4). Considering the requirement for formate in this mutant, the soluble enzyme of the wild type could be important in catalyzing glycine and methylenetetrahydrofolate formation. This possibility is discussed in the next section.

Possible Functions of the Mitochondrial and Soluble Isoenzymes

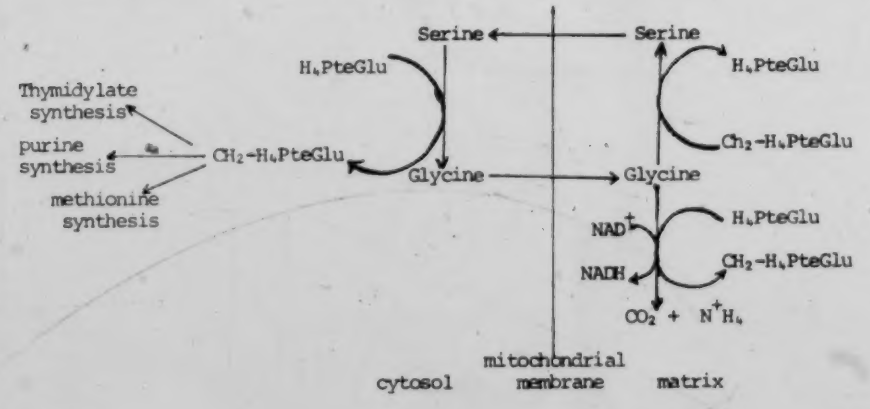
In the present study, serine hydroxymethyltransferase was associated with two different cellular fractions of *N. crassa* (Figs. 3, 4). The soluble isoenzyme was lacking in the *for* mutant (Tables 3, 4) and the mitochondrial isoenzyme favoured the formation of serine from glycine (Table 4). Cybulski and Fisher (1976) considered the physiological roles of the serine hydroxymethyltransferase of rat liver mitochrondria which had earlier been shown to exist as a matrix enzyme (Schnaitman and Greenawalt, 1968). In detailed transport studies, utilizing osmotic-swelling techniques, Cybulski and Fisher (1976) suggested that L-serine and glycine were readily transported across the inner mitochondrial membrane. However, an apparent lack of folate transport was also demonstrated.

Cytoplasmic and mitochondrial isoenzymes of malate dehydrogenase and glutamate-oxaloacetate transaminase have been implicated in the transfer of reducing equivalents across the mitochondrial inner membrane (*e.g.* Borst, 1963). Serine hydroxymethyltransferase, which catalyzes the production of a one-carbon unit, could conceivably be involved in the transport of potential one-carbon units across the inner membrane. In this regard, Cybulski and Fisher (1976), proposed such a system (Scheme 6A) for animal mitochondria. Formaldehyde, formed during intramitochondrial choline catabolism at the level of sarcosine and dimethylglycine oxidation, reacts non-enzymatically with tetrahydrofolate to form methylenetetrahydrofolate (Blakley, 1963). The mitochondrial serine hydroxymethyltransferase then transfers the C_1 unit to glycine, forming serine. This intramitochondrial serine is subsequently transported to the cytosol where it is converted to glycine by the cytosolic serine hydroxymethyltransferase. Methylenetetrahydrofolate, produced in the cytosol, thus serves as the one-carbon source necessary for a number of major biosyntheses, while the glycine, regenerated during the reaction, is transported back into the matrix to complete the cycle. Intramitochondrial glycine may also be cleaved, forming NH_3, CO_2, NADH and methylenetetrahydrofolate (Kikuchi, 1973).

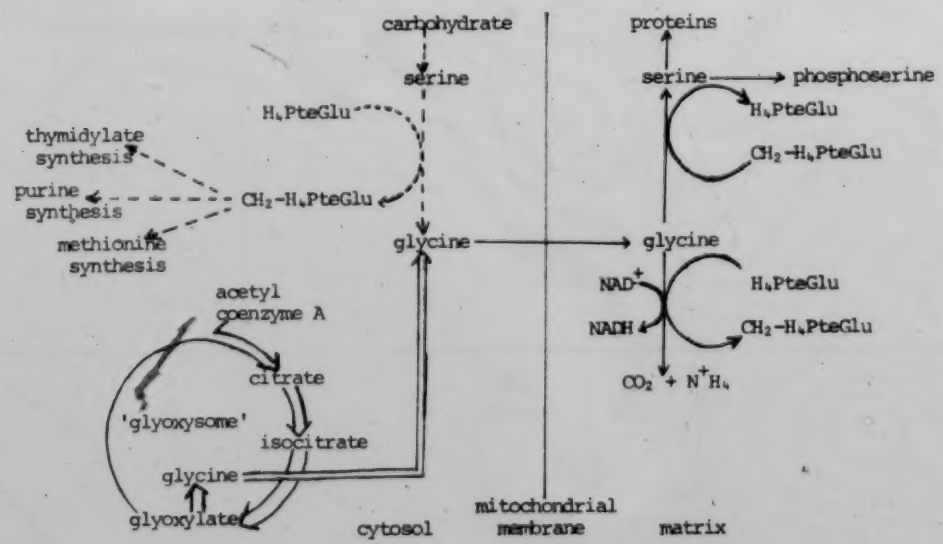
A somewhat different situation must prevail in *N. crassa* and this is summarized in Scheme 6B. In the wild type, glycine synthesis may be largely catalyzed by the soluble serine hydroxymethyltransferase as this isoenzyme favoured serine catabolism (Tables 3, 4). Conceivably, this reaction could have importance in providing C_1 units for the formation of thymidylate, purine and methionine. In this regard, the work of Cossins *et al.* (1976) showed that products of this nature were derived

SCHEME 6

A. Proposed one-carbon unit transport shuttle (quoted from Cybulski and Fisher, 1976)



B. Possible glycine-serine relationships in *N. crassa*, wild type and *for* mutant



Solid lines - reactions that are common to the wild type and *for* mutant.
Dashed lines - probable reactions occurring in wild type but not the *for* mutant.
Double lines - reaction that is more significant in the *for* mutant.

from [3-^{14}C]serine in the wild type. Glycine formed by the reaction might be partially transported into the mitochondria for the formation of serine in the presence of one-carbon units (Table 4). In the wild type, C_1 units for this may largely arise from glycine as cleavage to CO_2, NH_4^+ and one-carbon units is readily detected in the wild type (Cossins *et al.*, 1976), and this reaction is localized in the mitochondria of eucaryotes (Kikuchi, 1973).

The *for* mutant will be unable to generate cytosolic glycine by this route (Table 3) and glycine cleavage is of less importance to C_1 metabolism (Cossins *et al.*, 1976). In this case, glycine synthesis appears to occur mainly via the partial reactions of the glyoxylate cycle. It is clear, from Figures 5 and 6, that the mutant contains higher levels of isocitrate lyase activity and L-alanine:glyoxylate transaminase activity than the wild type. The [1-^{14}C]glyoxylate feeding experiments also show that the mutant incorporated more glyoxylate carbon into glycine than the wild type (Table 6). As this pathway would involve the glyoxysomes (Beevers, 1969), the glycine formed would presumably have to enter the mitochondria for serine formation to occur via the mitochondrial serine hydroxymethyltransferase. The required C_1 units for this latter synthesis could be derived from the formate supplement and in this regard it would be of interest to determine whether this mutant contains mitochondrial enzymes for methylenefolate production from this C_1 source.

The metabolic importance of intramitochondrial serine is not clear but conceivably this amino acid would be utilized in a variety of pathways, including those of phospholipid and protein biosynthesis within the mitochondrion.

Role of the Glyoxylate Cycle in Glycine Biosynthesis

The tricarboxylic acid cycle (TCA) functions in the majority of aerobic organisms (Krebs and Johnson, 1937) and allows complete oxidation of acetyl-CoA to CO_2 and water. The cycle also functions as a source of intermediates needed in the synthesis of more complex compounds. In the latter case, however, a large drain of cycle intermediates occurs. Replacement of these intermediates is generally accomplished by means of anaplerotic reactions of which the glyoxylate cycle is of major physiological importance.

Certain micro-organisms utilize acetate as a carbon source and as a source of energy. This situation has been documented for various bacterial species such as *E. coli*, *Pseudomonas*, and for autotrophs including algae and several higher plants (Kornberg, 1958, 1966; Harrop and Kornberg, 1966; John and Syrett, 1967; Yamamoto, 1961).

The intracellular localization of the two key cycle enzymes, namely isocitrate lyase and malate synthase, has received much attention. In bacterial systems they appear to be soluble (Kornberg, 1958), whereas in algae such as *Chorella vulgaris* there is some evidence (Harrop and Kornberg, 1966) that isocitrate lyase is particulate. In higher plants (Briedenbach and Beevers, 1967), as well as some nematodes (Patel and McFadden, 1977), the enzymes of the glyoxylate cycle are clearly located in a particulate structure now widely termed the glycoxysome.

Sjogren and Romano (1967) presented evidence for multiple forms of isocitrate lyase in *N. crassa*. The enzyme formed in acetate-grown cultures differed in several respects from that found in glucose-grown cells. For example, differences were found in pH optima, K_m values, and in sensitivity to phosphoenolpyruvate inhibition. Turian

(1961) found an active L-alanine:glyoxylate transaminase irrespective of carbon source in *Neurospora* and *Allomyces* and he suggested that isocitrate lyase, formed in glucose-grown cells, may play a role in the biosynthesis of glycine. In addition, McCurdy and Cantino (1960) demonstrated that isocitrate lyase and a glycine-alanine transaminase are formed concomitantly in synchronously developing sporangial plants of *Blastocladiella emersonii*, and have ascribed a morphogenetic role to the former enzyme.

It is now acknowledged that glucose represses enzymes of the glyoxylate cycle. For example, isocitrate lyase formation has been studied in this regard (Kornberg, 1966). In the present work considerable isocitrate lyase activity was detected in both the wild type and the *for* mutant when sucrose was present in the growth media (Fig. 5). Figures 5 and 6 show that the activity of this enzyme and of L-alanine: glyoxylate transaminase were highest in the mutant. In agreement with this situation, the [1-^{14}C]glyoxylate feeding experiments confirmed the suspicion that the glyoxylate cycle could be responsible for glycine synthesis particularly in the mutant (Table 6). The feeding experiments also suggest that for the wild type there is an extensive conversion of glycine to serine, while in the *for* mutant, glycine is the main product formed from glyoxylate (Table 6). Moreover, considerably higher levels of radioactivity were found in aspartic acid for the wild type (Table 6). This suggests that in the wild type glyoxylate metabolism leads to the formation of cycle organic acids with oxaloacetate supporting aspartate production. The higher levels of malate synthase activity in the wild type support this possibility (Table 5).

The *for* mutant, in lacking the soluble serine

hydroxymethyltransferase (Table 3) will be deficient in glycine and this has been confirmed by direct assay of this amino acid (Cossins *et al.*, 1976). It is possible that this condition may have a stimulatory effect on isocitrate lyase activity as it is reasonable to assume that diversion of glyoxylate from the cycle will be controlled. It is also possible, as noted earlier, that glycine formed from glyoxylate in the glyoxysome would be, to some extent, transported to the mitochondria for the formaton of serine. The relationships between serine synthesis and glyoxylate formation in this species need to be evaluated and represent an interesting area for future work.

McFadden and Howes (1962) have studied the properties of isocitrate lyase from *Pseudomonas indigofera*. They found that acetate, L-alanine, L-aspartate, butyrate, citrate, L-glutamate and glycine had little or no effect on activity when present at 8.3×10^{-3}M. It is of some interest that glycine was without effect but also that serine was not examined. In *Brevibacterium flavum* isocitrate lyase was competitively inhibited by oxalate, glyoxylate, malate, oxaloacetate and L-ketoglutarate, but no data for glycine or serine were reported (Ozaki and Shiio, 1968). Perhaps different controls operate in eukaryotes or conceivably actively growing systems could have controls that modulate enzyme synthesis.

Concluding Remarks

Two apparent isoenzymes of serine hydroxymethyltransferase have been fractionated from the cytoplasm of the wild type and the mitochondria of the formate-requiring mutant. A similar partition of this enzyme has also been reported for *S. cerevisiae* wild type (Zelikson and Luzzati, 1976). The mitochondrial isoenzyme of *N. crassa* appears

to favour serine production from glycine. Furthermore, the absence of the soluble enzyme in the *for* mutant appears to necessitate glycine biosynthesis by the partial reactions in the glyoxylate cycle. From the present data it follows that the soluble enzyme is mainly concerned with glycine and one-carbon unit biosynthesis. Further research on the metabolic functions of the mitochondrial serine hydroxymethyltransferase appear warranted and might be complemented by work on mutants lacking this particulate isoenzyme.

BIBLIOGRAPHY

AKHTAR, M., EL-OBEID, H.A. and JORDAN, P.M. 1975. Mechanistic, inhibitory and stereochemical studies on cytoplasmic and mitochondrial serine hydroxymethylases. *Biochem. J.* 145: 159-168.

ALEXANDER, N. and GREENBERG, D.M. 1956. Studies on the purification and properties of the serine-forming enzyme. *J. Biol. Chem.* 220: 775-785.

ANDREWS, P. 1964. Estimation of the molecular weights of proteins of Sephadex gel filtration. *Biochem. J.* 91: 222-233.

BASSETT, R., WEIR, D.G. and SCOTT, J.M. 1976. The identification of hexa-, hepta- and octo-glutamates as the polyglutamyl forms of folate found throughout the growth cycle of yeast. *J. Gen. Microbiol.* 93: 169-172.

BAUGH, C.M., BRAVERMAN, E. and NAIR, M.G. 1975. In: *Chemistry and Biology of Pteridines.* (W. Pfleiderer, ed.), pp. 465-474. Walter de Gruyter, Berlin.

BEEVERS, H. 1969. Glyoxysomes of castor bean endosperm and their relation to gluconeogenesis. *Ann. N.Y. Acad. Sci.* 168: 313-324.

BEREMAND, P.D. and SOJKA, G.A. 1977. Mutational anlaysis of serine-glycine biosynthesis in *Rhodopseudomonas capsulata*. *J. Bacteriol.* 130 (No. 1): 532-534.

BLAKLEY, R.L. 1963. The biosynthesis of thymidylic acid. IV. Further studies on thymidylate synthetase. *J. Biol. Chem.* 238: 2113-2118.

BLAKLEY, R.L. 1969. In: *The Biochemistry of Folic Acid and Related Pteridines*. pp. 291-293. American Elsevier Publ. Co. Inc. John Wiley & Sons, Inc., New York.

BORST, P. 1963. Interrelations between cytoplasmic and mitochondrial diphosphoryridine nucleotide in Ehrlich ascites tumour cells. *Proc. Int. Congr. Biochem.* 5th, 1961(2), 233-247.

BOTSFORD, J.L. Jr. and PARKS, L.W. 1969. Serine transhydroxymethylase in methionine biosynthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 97: 1176-1183.

BRIEDENBACH, R.W. and BEEVERS, H. 1967. Association of the glyoxylate cycle enzymes in a noval subcellular particle from castor bean endosperm. *Biochem. Biophys. Res. Commun.* 27: 462-469.

BRUIN, W.J., NELSON, E.B. and TOLBERT, N.E. 1970. Glycolate pathway in green algae. *Plant Physiol.* 46: 386-391.

BURTON, E.G. and METZENBERG, R.L. 1975. Regulation of methionine biosynthesis in *N. crassa*. *Arch. Biochem. Biophys.* 168: 219-229.

BURTON, E.G. and SAKAMI, W. 1969. The formation of methionine from the monoglutamate form of methyl-tetrahydrofolate by higher plants. *Biochem. Biophys. Res. Commun.* 36: 228-234.

BYERRUM, R.V., RINGLER, R.L., HAMILL, R.L. and BALL, C.D. 1955. Serine and formaldehyde as metabolic precursors for the nicotine N-methyl group. *J. Biol. Chem.* 216: 371-378.

CAMP, R.R. and WHITTINGHAM, W.F. 1972. Host-parasite relationships in sooty blotch disease of white clover. *Am. J. Bot.* 59: 1057-1067.

CAMPBELL, L.L. 1955. Transamination of amino acids with glyoxylic

acid in bacterial extracts. *J. Bacteriol.* 71: 81-83.

CANTINO, E.C. 1960. Relations of metabolism to cell development. In: Handb. d. Pflanzenphysiologie, XV, W. Ruhland, ed. Springer-Verlag, Heidelberg, Germany (in press).

CANTINO, E.C. and HORENSTEIN, E.A. 1959. The stimulatory effect of light upon growth and CO_2 fixation in *Blastocladiella*. III. Further studies, *in vivo* and *in vitro*. *Physiol. Plant.* 12: 251-263.

CANVIN, D.T. and BEEVERS, H. 1961. Sucrose synthesis from acetate in the germinating castor bean: kinetics and pathway. *J. Biol. Chem.* 236: 988-995.

CHASIN, L.A., FELDMAN, A., KONSTRAM, M. and URLAUB, G. 1974. Reversion of a Chinese Hamster cell auxotrophic mutant. *Proc. Nat. Acad. Sci. U.S.A.* 71: 718-722.

CLANDININ, M.T. and COSSINS, E.A. 1972. Localization and interconversion of tetrahydropteroylglutamates in isolated pea mitochondria. *Biochem. J.* 128: 29-40.

COMBEPINE, G. 1969. Etude comparative de la transaminase alanine-glyoxylate et malate synthetase chez *Neurospora crassa*. *Bull. Soc. Chim. Biol.* 51: 1309-1317.

COMBEPINE, G., COSSINS, E.A. and CHAN, P.Y. 1975. In: *Chemistry and Biology of Pteridines*. (Pfleiderer, W., ed.), pp. 331-339. Walter de Gruyter, Berlin.

COSSINS, E.A. 1978. One-carbon metabolism. In: *The Biochemistry of Plants*. Vol. 2. (Stumpf, P.K. and Conn, E.E., eds.), Academic Press, N.Y. (in press).

COSSINS, E.A., CHAN, P.Y. and COMBEPINE, G. 1976. One carbon metabolism

in *Neurospora crassa* wild type and in mutants partially deficient in serine hydroxymethyltransferase. *Biochem. J.* 160: 305-314.

COSSINS, E.A. and SINHA, S.K. 1964. The interconversion of glycine and serine by plant tissue extracts. *Biochem. J.* 101: 542-549.

COSSINS, E.A. and SINHA, S.K. 1965. Occurrence and properties of L-amino acid: 2-glyoxylate aminotransferase in plants. *Can. J. Biochem.* 43: 685-698.

CURTHOYS, N.P. and RABINOWITZ, J.C. 1971. Formyltetrahydrofolate synthetase: binding of folate substrates and kinetics of the reverse reaction. *J. Biol. Chem.* 247: 1965-1971.

CYBULSKI, R.L. and FISHER, R.R. 1976. Intramitochondrial localization and proposed metabolic significance of serine transhydroxymethylase. *Biochemistry* 15 (No. 15): 3183-3187.

DAVIS, R.H. and DeSERRES, F.J. 1970. Genetic and microbiological research techniques for *N. crassa*. *Methods in Enzymol.* 17: 79-143.

DICKERMAN, H.W., STEARS, E.J., REDFIELD, B.G. and WEISSBACH, H. 1967. Methionyl soluble ribonucleic acid transformylase. I. Purification and partial characterization. *J. Biol. Chem.* 242: 1522-1525.

DIXON, G.H. and KORNBERG, H.L. 1959. Assay methods for key enzymes of the glyoxylate cycle. *Biochem. J.* 72: 3P.

DODD, W.A. and COSSINS, E.A. 1970. Homocysteine-dependent transmethylases catalyzing the synthesis of methionine in germinating pea seeds. *Biochim. Biophys. Acta* 201: 461-470.

DUNTZE, W., NEUMANN, D., GANCEDO, J.M., ATZPODIEN, W. and HOLZER, H. 1969. Studies on the regulation and localization of the glyoxylate cycle enzymes in *S. cerevisiae*. *Eur. J. Biochem.* 10: 83-89.

FLAKS, J.G. and COHEN, S.S. 1959. Virus-induced acquisition of metabolic function. I. Enzymatic formation of 5-hydroxymethyl deoxy-cytidylate. *J. Biol. Chem.* 234: 1501-1506.

FLAVELL, R.B. and FINCHAM, J.R.S. 1968. Acetate-nonutilizing mutants of *Neurospora crassa*. II. Biochemical deficiencies and the role of certain enzymes. *J. Bacteriol.* 95: 1963-1968.

FOO, S.K., BADOUR, S.S. and WAYGOOD, E.R. 1971. Regulation of isocitrate lyase from autotrophic and photoheterotrophic cultures of *Gloeomonas* sp. *Can. J. Bot.* 49: 1647-1653.

FUJIOKA, M. 1969. Purification and properties of serine hydroxymethyl-ase from soluble and mitochondrial fractions of rabbit liver. *Biochim. Biophys. Acta* 185: 338-349.

GOLDSWORTHY, P.D., WINNINCK, T. and GREENBERG, D.M. 1949. Distribution of ^{14}C in glycine and serine of liver protein following the administration of labeled glycine. *J. Biol. Chem.* 180: 341-343.

GRAVES, L.B. Jr., ARMENTROUT, V.N. and MAXWELL, D.P. 1976. Distribution of glyoxylate-cycle enzymes between microbodies and mitochondria in *Aspergillus tamarii*. *Planta* (Berl.) 132: 143-148.

GRAVES, L.B. Jr., TRELEASE, R.N., GRILL, A. and BECKER, W.M. 1972. Localization of glyoxylate cycle enzymes in glyoxysomes in *Euglena*. *J. Protozool.* 19: 527-532.

GREEN, A.A. and HUGHES, W.L. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents.

Methods in Enzymol. 1: 67-89.

GREENE, R.C. and RADOVICH, C. 1975. Role of methionine in the regulation of serine hydroxymethyltransferase in *Escherichia coli*. *J. Bacteriol.* 124: 269-278.

HALBREICH, A. and RABINOWITZ, M. 1971. Isolation of *S. cerevisiae* mitochondrial formyltetrahydrofolic acid: Methionyl-tRNA transformylase and the hybridization of mitochondrial fMET-tRNA with mitochondrial DNA. *Proc. Nat. Acad. Sci. U.S.A.* 68: 294-298.

HARROLD, C.E. and FLING, M. 1952. Two mutants of *N. crassa* which utilize formate or formaldehyde for growth. *J. Biol. Chem.* 194: 399-406.

HARROP, L.C. and KORNBERG, H.L. 1966. The role of isocitrate lyase in the metabolism of algae. *Proc. Roy. Soc. B.* 166: 11-29.

HARVEY, R.J. and DEV, I.K. 1974. Regulation in the folate pathway of *Escherichia coli*. In: *Advances in Enzyme Regulation.* Pergamon Press, Vol. 13, pp. 99-124.

HAUSCHILD, H.H. 1959. The interconversion of glycine and serine in *Zea mays*. *Can. J. Biochem. Physiol.* 37: 887-894.

HIATT, A.J. 1961. Preparation and some properties of soluble succinic dehydrogenase from higher plants. *Plant Physiol.* 36: 552-557.

HORIKOSHI, K. and DOI, R.H. 1967. Synthesis of N-formyl methionyl-sRNA by *Bacillus subtilis* extracts. *Arch. Biochem. Biophys.* 122: 685-693.

JOHN, P.C.L. and SYRETT, P.J. 1967. The purification and properties of isocitrate lyase from *Chlorella*. *Biochem. J.* 105: 409-416.

JUNGERMANN, K., THAUER, R.K. and DECKER, K. 1968. The synthesis of

one-carbon units from CO_2 in *Clostridium kluyveir*. *Eur. J. Biochem.* 3: 351-359.

KERWAR, S.S., MANGUM, J.H., SCHRIMGEOUR, K.G., BRODIE, J.D. and HUENNEKENS, F.M. 1966. Interrelationship of adenosyl methionine and methyl-B_{12} in the biosynthesis of methionine. *Arch. Biochem. Biophys.* 116: 305-318.

KIKUCHI, G. 1973. The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.* 1: 169-187.

KIKUCHI, G., SHEMIN, D. and BACHMAN, J. 1958. The enzymatic synthesis of α-aminolevulinic acid. *Biochim Biophys. Acta* 28: 219-220.

KISAKI, T., IMAI, A. and TOLBERT, N.E. 1971. Intracellular localization of enzymes related to photorespiration in green leaves. *Plant Cell Physiol.* 12: 267-273.

KOBR, M.J., TURIAN, G. and ZIMMERMAN, E.J. 1965. Changes in enzymes regulating isocitrate breakdown in *Neurospora crassa*. *Arch. Mikrobiol.* 52: 169-177.

KOBR, M.J., VANDERHAEGHE, F. and COMBEPINE, G. 1969. Particulate enzymes of the glyoxylate cycle in *Neurospora crassa*. *Biochem. Biophys. Res. Commun.* 37: 640-645.

KORNBERG, H.L. 1958. The metabolism of C_2 compounds in microorganisms. I. The incorporation of [2-^{14}C]acetate by *Pseudomonas flueorescens*, and by a corynebacterium, grown on ammonium acetate. *Biochem. J.* 68: 535-542.

KORNBERG, H.L. 1966. The role and control of the glyoxylate cycle in *E. coli*. *Biochem. J.* 99: 1-11.

KORNBERG, H.L. and KREBS, H.A. 1957. Synthesis of cell constituents

from C_2 units by a modified tricarboxylic acid cycle. *Nature* 179: 988-991.

KREBS, H.A. and JOHNSON, W.A. 1937. The role of citric acid in intermediate metabolism in animal tissues. *Enzymologia* 4: 148-156.

KUNTZEL, H. and NOLL, H. 1967. Mitochondrial and cytoplasmic polysomes from *N. crassa*. *Nature* (London) 215: 1340-1345.

LaBORDA, F. and MAXWELL, D.P. 1976. Ultrastructural changes in *Cladosporium cucumerinum* during pathogenesis. *Can. J. Microbiol.* 22: 394-403.

LJUNGDAHL, L.G. and WOOD, H.G. 1969. Total synthesis of acetate from CO_2 by heterotrophic bacteria. *Ann. Rev. Microbiol.* 23: 515-538.

LOR, K.L. and COSSINS, E.A. 1972. Regulation of C_1 metabolism by L-methionine in *S. cerevisiae*. *Biochem. J.* 130: 773-783.

LOR, K.L. and COSSINS, E.A. 1978. Relationships between glycollate and folate metabolism in *Euglena gracilis*. *Phytochem.* 17: 659-665.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

MACKINNEY, G., CHICHESTER, C.O. and NAKAYAMA, T. 1955. The incorporation of glycine carbon into β-carotene in *Phycomyces blakesleeanus*. *Biochem. J.* 60: 37-38.

MALEY, F. 1962. Nucleotide interconversions. VI. The enzymic formation of 5-methyluridylic acid. *Arch. Biochem. Biophys.* 96: 550-556.

MAXWELL, D.P., MAXWELL, M.D., HÄNSSLER, G., ARMENTROUT, V.N., MURRAY, G. M. and HOCH, H.C. 1975. Microbodies and glyoxylate-cycle

enzyme activities in filamentous fungi. *Planta* (Berl.) 124: 109-123.

MAZELIS, M. AND LIU, E.S. 1967. Serine transhydroxymethylase of cauliflower (*Brassica oleracea* var. *botrytis* L.): Partial purification and properties. *Plant Physiol.* 42: 1763-1768.

MCCURDY, H.D. Jr. and CANTINO, E.C. 1960. Isocitritase, glycine-alanine transaminase, and development in *Blastocladiella emersonii*. *Plant Physiol.* 35: 463-476.

McFADDEN, B.A. and HOWES, W.V. 1962. Isocitrate lyase and malate synthase in *Pseudomonas indigofera*. *J. Bacteriol.* 84: 1216-1221.

MILLER, B.A. and NEWMAN, E.B. 1973. Control of serine transhydroxymethylase in *Escherichia coli* K12. *Can. J. Microbiol.* 20: 41-47.

MILLS, G.L. and CANTINO, E.C. 1975. Isolation and characterization of microbodies and symphyomicrobodies with different buoyant densities from the fungus *Blastocladiella emersonii*. *Biochem. Biophys. Res. Commun.* 67: 1256-1263.

MÜLLER, M., HOGG, J.F. and DeDUVE, C. 1968. Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes in *Tetrahymena pyriformis*. *J. Biol. Chem.* 243: 5385-5395.

NAKANO, Y., FUJIOKA, M. and WADA, H. 1968. Studies on serine hydroxymethylase isoenzymes from rat liver. *Biochim. Biophys. Acta* 159: 19-26.

NASH, T. 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* 55: 416-421.

NEWMAN, E.B., MILLER, B. and KAPOOR, V. 1974. Biosynthesis of single carbon units in *Escherichia coli* K12. *Biochim. Biophys. Acta* 338: 529-539.

NOWAK, T. and HIMES, R.H. 1971. Formyltetrahydrofolate synthetase: The role of the sulfhydryl gouprs. *J. Biol. Chem.* 246: 1285-1293.

OGUR, M., LIU, T.N., CHEUNG, I., PAULAVICIUS, I., WALES, W., MEHNERT, D. and BLAISE, D. 1977. "Active" one-carbon generation in *Saccharomyces cerevisiae*. *J. Bacteriol.* 129: 926-933.

OSBORNE, M.J., TOLBERT, P.T. and HUENNEKENS, F.M. 1960. The structure of "active formaldehyde" (N^5, N^{10}-methylenetetrahydrofolic acid). *J. Am. Chem. Soc.* 82: 4921-4927.

OZAKI, H. and SHIIO,I. 1968. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. I. Inhibition of isocitrate lyase and isocitrate dehydrogenase by organic acids related to the TCA and glyoxylate cycles. *J. Biochem.* 64(3): 355-363.

PALEKAR, A.G., TATE, S.S. and MEISTER, A. 1973. Rat liver aminomalonate decarboxylase. *J. Biol. Chem.* 248: 1158-1167.

PANG, S. and COSSINS, E.A. 1978. Glycine biosynthesis in a *Neurospora* mutant deficient in cytosolic serine hydroxymethyltransferase. *Can. Soc. Plant Physiol. Proc.* 18: 29.

PARKER, D.J., WU, T-F. and WOOD, H.G. 1971. Total synthesis of acetate from CO_2, methyltetrahydrofolate, an intermediate, and a procedure for separation of the folates. *J. Bacteriol.* 108: 770-776.

PATEL, T.R. and McFADDEN, B.A. 1977. Particulate isocitrate lyase and malate synthetase in *Canorhabditis elegans*. *Arch. Biochem.*

Biophys. 193: 24.

PLATZER, E.G. and CAMPUZANO, H.C. The serine hydroxymethyltransferase of *Plasmodium lophurae*. *J. Protozool.* 23(2): 282-286.

PRATHER, C.W. and SISLER, E.C. 1966. Purification and properties of serine hydroxymethyltransferase from *Nicotiana rustica* L. *Plant and Cell Physiol.* 7: 457-463.

RABINOWITZ, J.C. and PRICER, W.E. Jr. 1957. Formation, isolation and properties of 5-formiminotetrahydrofolic acid. *Fed. Proc.* 16: 236.

RABINOWITZ, J.C. and PRICER, W.E. Jr. 1958. Crystallization of tetrahydrofolic formylase. *Fed. Proc.* 17: 293.

RABINOWITZ, J.C. and PRICER, W.E. Jr. 1962. Formyltetrahydrofolate synthetase. I. Isolation and crystallization of the enzyme. *J. Biol. Chem.* 237: 2898-2902.

ROGGENKAMP, R., SAHM, H., HINKELMANN, W. and WAGNER, F. 1975. Alcohol oxidase and catalase in peroxisomes of methanol-grown *Candida boidinii*. *Eur. J. Biochem.* 59: 231-236.

SAKAMI, W. 1948. The conversion of formate and glycine to serine and glycogen in the intact rat. *J. Biol. Chem.* 176: 995-996.

SCHERTEL, M.E., BOEHNE, J.W. and LIBBY, D.A. 1965. Folic acid derivatives in yeast. *J. Biol. Chem.* 240: 3154-3158.

SCHNAITMAN, C. and GREENAWALT, J.W. 1968. Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* 38: 158-175.

SEIGEL, I. and LAFAYE, T. 1950. Formation of the β-carbon of serine from formaldehyde. *Proc. Soc. Exptl. Biol. Med.* 74: 620-623.

SELHUB, J., BURTON, E. and SAKAMI, W. 1969. Identification of three

enzymes specifically involved in the *de novo* methionine methyl biosynthesis of *N. crassa*. *Fed. Proc. Fed. Am. Soc. Exptl. Biol.* 28: 352.

SHANE, B. and STOKSTAD, E.L.R. 1977. Metabolism of 5-methyltetrahydrofolate by *Lactobacillus casei*. *J. Gen. Microbiol.* 103: 249-259.

SHEMIN, D. 1956. The biosynthesis of porphyrins: the succinate-glycine cycle. In: *Currents in Biochemical Research*. (Green, D.E., ed.) pp. 518-536. Interscience Publishers, New York.

SHEMIN, D. 1964. The biological conversion of L-serine to glycine. *J. Biol. Chem.* 162: 297-307.

SIEKEVITZ, P. and GREENBERG, D.M. 1949. The biological formation of serine from glycine. *J. Biol. Chem.* 180: 845-856.

SJOGREN, R.E. and ROMANO, A.H. 1967. Evidence for multiple forms of isocitrate lyase in *Neurospora crassa*. *J. Bacteriol.* 93: 1638-1643.

STAUFFER, G.V., BAKER, C.A. and BRENCHLEY, J.E. 1974. Regulation of serine transhydroxymethylase activity in *Salmonella typhimurium*. *J. Bacteriol.* 120: 1017-1025.

SZABO, A.S. and AVERS, C.J. 1969. Some aspects of regulation of peroxisomes and mitochondrial in yeast. *Ann. N.Y. Acad. Sci.* 168: 302-312.

TABOR, H. and WYNGARDEN, L. 1959. The enzymatic formation of formiminotetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 10-formyltetrahydrofolic acid in the metabolism of formiminoglutamic acid. *J. Biol. Chem.* 234: 1830-1846.

TADA, K., NARISAWA, K., YOSHIDIA, T., KONNO, T., YOKOYAMA, Y., NAKAGAWA, H., TANNO, K., MOCHIJAHI, K., ARAKAWA, T., YOSHIDA, T. and

KIKUCHI, G. 1969. Hyperglucinemia: a defect in glycine cleavage reaction. *Tohoku J. Exp. Med.* 98: 289-296.

TAYLOR, R. and WEISSBACH, H. 1965. Radioactive assay for serine transhydroxymethylase. *Anal. Biochem.* 13: 80-84.

TOLBERT, N.E. 1962. Glycolate pathway in photosynthetic mechanisms in green plants. *Nat. Acad. Sci. Nat. Res. Council Publ.* No. 1145, pp. 648-662.

TOLBERT, N.E. 1971. Microbodies-peroxisomes and glyoxysomes. *Ann. Rev. Plant Physiol.* 22: 45-74.

TOLBERT, N.E., OESER, A., KISAKI, T., HAGEMAN, R.H. and YAMAZAKI, R.K. 1968. Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. *J. Biol. Chem.* 243: 5179-5184.

TOLBERT, N.E., OESER, A., YAMAZAKI, R.K., HAGEMAN, R.H. and KISAKI, T. 1969. A survey of plants for leaf peroxisomes. *Plant Physiol.* 44: 135-147.

TURIAN, G. 1961. Cycle glyoxylique, transaminase alanine-glyoxylate et differenciation sexuelle chez *allomyces* et *Neurospora*. *Pathol. Microbiol.* 24: 819-839.

VAN DYKE, K., TREMBLAY, G.C., LANTZ, C.H. and SZUSTKIEWICZ, C. 1970. The source of purines and pyrimidines in *Plasmodium berghei*. *Am. J. Trop. Med. Hyg.* 19: 202-208.

VOGEL, J.H. 1964. Distribution of lysine pathways among fungi: evolutionary implications. *Am. Naturalist* 98:435-446.

WEISSBACH, H., PETERKOFSKY, A., REDFIELD, B.C. and DICKERMAN, H.J. 1963. Studies on the terminal reaction in the biosynthesis of methionine. *J. Biol. Chem.* 238: 3318-3324.

WERGIN, W.P. 1972. Ultrastructural comparison of microbodies in pathogenic and saprophytic hyphae of *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* 62: 1045-1051.

WHITTAKER, V.K. and BLAKLEY, R.L. 1961. The biosynthesis of thymidylic acid. II. Preliminary studies with calf thymus extracts. *J. Biol. Chem.* 236: 838-841.

WHITTAKER, V.K. and BLAKLEY, R.L. 1977. Rate limiting steps in folate metabolism by *Lactobacillus casei*. *J. Gen. Microbiol.* 103: 261-270.

WILKINSON, A.P. and DAVIES, D.D. 1958. Serine-glycine interconversion in plant tissues. *Nature* 181: 1070-1071.

WINNICK, T., MORING-CLAESSON, I. and GREENBERG, D.M. 1948. Distribution of radioactive carbon among certain amino acids of liver homogenate protein, following uptake experiments with labeled glycine. *J. Biol. Chem.* 175: 127-132.

WRIGHT, B.E. 1951. Utilization of glyoxylic and glycolic acids by a *Neurospora* mutant requiring glycine or serine. *Arch. Biochem. Biophys.* 31: 332-333.

YAMAMOTO, Y. 1961. A metabolic pathway of isocitric acid in cotyledons of a bean, *Vigna sesquipedalis*. *Plant and Cell Physiol.* 2: 277-289.

YOSHIDA, T., KIKUCHI, G., TADA, K., NARISAWA, K. and ARAKAWA, T. 1969. Physiological significance of glycine cleavage system in human liver as revealed by the study of a case of hyperglycinemia. *Biochem. Biophys. Res. Commun.* 35: 577-583.

YOSHIDA, T. and KIKUCHI, G. 1970. Major pathways of glycine and serine catabolism in rat liver. *Arch. Biochem. Biophys.* 139: 380-392.

ZELIKSON, R. and LUZZATI, M. 1976. Two forms of serine transhydroxymethylase, one absent in a thymidylate-less mutant in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 64: 7-13.